NATIONAL INSTITUTE OF HEALTH

# JOURNAL OF THE NATIONAL CANCER INSTITUTE

VOLUME 3 *August 1942* NUMBER 1

FEDERAL SECURITY AGENCY
UNITED STATES PUBLIC HEALTH SERVICE
WASHINGTON, D. C.

# CONTENTS


| | Page |
|---|---|
| Effect of Carcinogens on Small Organisms. IV. Adaptation of Bacteria to High Temperatures, by R. R. Spencer and M. B. Melroy | 1 |
| Comparative Enzymatic Activity of Transplanted Hepatomas and of Normal, Regenerating, and Fetal Liver, by Jesse P. Greenstein, Jesse E. Edwards, Howard B. Andervont, and Julius White | 7 |
| Induction of Cirrhosis of the Liver and of Hepatomas in Mice With Carbon Tetrachloride, by Jesse E. Edwards and Albert J. Dalton | 19 |
| Effect of Supplementary Methionine or Choline Plus Cystine on the Incidence of *p*-Dimethylaminoazobenzene-Induced Hepatic Tumors in the Rat, by Julius White and Jesse E. Edwards | 43 |
| Sulfhydryl Groups in Normal and Tumorous Hepatic Tissue Extracts Before and After the Addition of Salts, by Jesse P. Greenstein | 61 |
| Genetic Analysis of Susceptibility to Induced Pulmonary Tumors in Mice, by W. E. Heston | 69 |
| Inheritance of Susceptibility to Spontaneous Pulmonary Tumors in Mice, by W. E. Heston | 79 |
| Limits of Accuracy in Experimental Carcinogenesis as Exemplified by Tumor Induction With Ultraviolet Radiation, by Harold F. Blum, Hugh G. Grady, and John S. Kirby-Smith | 83 |
| Relationships Between Dosage and Rate of Tumor Induction by Ultraviolet Radiation, by Harold F. Blum, Hugh G. Grady, and John S. Kirby-Smith | 91 |
| Effect of Climatic Environment Upon the Genesis of Subcutaneous Tumors Induced by Methylcholanthrene and Upon the Growth of a Transplantable Sarcoma in C3H Mice, by Edward W. Wallace, Helene M. Wallace, and Clarence A. Mills | 99 |
| Photometric Histochemical Determination of Thymonucleic Acid in Experimental Epidermal Carcinogenesis, by R. E. Stowell | 111 |
| Genetics of the Susceptibility of Mice to a Transplantable Melanoma, by Juliet M. Spangler, J. M. Murray, and C. C. Little | 123 |

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# Effect of Carcinogens on Small Organisms. IV. Exposure of Bacteria to High Temperatures

By R. R. SPENCER, *assistant chief*, and M. B. MELROY, *assistant bacteriologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

In the first paper of this series (*1*) we recorded the results of the action of a carcinogenic agent, methylcholanthrene, upon a well-known species of bacteria, *Eberthella typhi*. No structural, functional, or immunological changes were observed after *continuous* exposure of the organism to this agent for 240 consecutive daily transfers. It was noticed, however, (1) that the amount of visible growth in approximately the latter 40 vials of the methylcholanthrene-adapted series was considerably less than that in the vials of the first part of the series or of any of the vials of the untreated control series, as determined by the degree of turbidity, and (2) that a much larger amount of inoculum from the latter tubes of the methylcholanthrene - treated series was needed in order to obtain a good growth and distribution of colonies on agar slants.

This observation was not regarded as significant until subsequent tests upon paramecia (*2, 3*) revealed that a continuous exposure to methylcholanthrene (1γ per cubic centimeter) resulted in (1) an increased capacity to survive as compared with control strains. This phenomenon was not manifested until after 350 days' exposure; (2) an increased capacity to reach higher population levels, appearing after 450 days' exposure; and (3) a disappearance of both of the above-mentioned patterns of behavior after 750 days' exposure, and, when compared with numerous control strains, a definite weakening of the species as measured by the ability of the organisms to survive and as measured by population levels.

The third observation, the weakened condition of the species, has not yet been reported in detail and awaits the results of a further continuation of this uninterrupted, exposure of the strain to methylcholanthrene.

In our hands the results of continuous exposure of paramecia to both carcinogenic and noncarcinogenic hydrocarbons have not only revealed a final weakening but also in some cases the death of the

472192—42——1

species, provided the exposure was uninterrupted over a long period of continuous cell-division cycles.

That the same environment which is apparently harmless to individual organisms for a number of successive generations may be finally fatal to the species has now been demonstrated on two different species of bacteria. Bacteria were selected for these tests because of the rapidity of cell division and because we have now developed a simple method of transferring bacterial cultures which seems to exclude completely the possibility of air-borne contaminations.

## MATERIALS AND METHODS

Into 15-ml.-capacity pyrex-glass vaccine vials are placed 5 or 6 ml. of plain sugar-free broth. Each vial is fitted with a rubber stopper which has a central depression or moat with a thin diaphragm at the bottom of the moat for the ready penetration of a hypodermic needle. The rubber stoppers are loosely fitted into place with a short cotton thread between the stopper and the mouth of the vial. Without this precaution the stoppers will be blown out when autoclaved at 15 pounds pressure for one-half hour. When removed from the autoclave and still warm, the cotton threads are withdrawn and the stoppers tightly fitted. After cooling, there is usually a slightly negative pressure in each vial. This helps to preserve an air-tight seal. The vials are then incubated at 37° C. for 7 days. In this way we have prepared about a thousand vials of sterile broth, and no contaminations have occurred.

One-milliliter-capacity tuberculin syringes with 26-gage needles are employed for the inoculation and transfer of bacteria from vial to vial. The small-gage needles prevent the thin rubber diaphragm from being torn. The syringes and needles are cleaned with tap water and 95 percent alcohol, thoroughly dried, placed in large test tubes with cotton stoppers, and autoclaved between usages.

This method of serial transfer is believed to be exceptionally free from the possibility of air-borne contaminations and appears to be equally as safe as our pyrex-glass-box technique described elsewhere (*4*). In fact, the method was tested by setting up 2 series of 100 vials each containing sterile broth, and each day for 100 consecutive days one-half milliliter of broth was transferred by means of the sterile syringes from 1 vial to the next in series. After 7 days' incubation, no vials of either series displayed any growths. In addition, a single vial was penetrated each day for 30 consecutive days and one-half milliliter of broth was withdrawn completely and re-injected into the vial. In this case no precaution was taken to swab the stopper with tincture of iodine which is our custom when cultures are transferred. No growth appeared in this vial after the 30-day period and after 7 days' further incubation although the broth was capable of growing *Escherichia coli* with which it was finally inoculated.

## RESULTS

### Streptococcus Hemolyticus (N. Y. No. 5 Strain)

The optimum temperature at which *Streptococcus hemolyticus* (N. Y. No. 5. strain) grows is 37° C. Attempts were made to grow it continuously in series at 42°. Transfers were made daily, and each fresh sterile vial was heated for 20 minutes at 42° before receiving one-half milliliter of inoculum from the previous vial in the series, in order to insure that the organisms were never exposed to a lower temperature.

After 24 hours' incubation at 42°, however, and after the inoculation of the next vial in series, each vial was then incubated at 37° for 2 or 3 days and finally stored at room temperature. In this way, the continuously developing species never experienced a temperature lower than 42°.

It was found that the streptococcus grew equally as well at 42° C. as the control series at 37° for five consecutive transfers. The sixth transfer, however, did not grow, although it was inoculated with one-half milliliter of inoculum from vial No. 5. The test was repeated, and again the first five vials yielded a good growth while the sixth vial failed to grow. There was no gradual diminution of the growth as measured by the turbidity. The fifth vial appeared equally as turbid as the first four. No morphologic changes were seen when stained preparations were examined microscopically.

When vial No. 5, the last to show growth, had aged 7 days (1 day at 42° C., 3 days at 37°, and 3 days at room temperature), a transfer was again made to a fresh vial which was incubated at 37°. The second vial in this new series was also incubated at 37° in order to provide a rest period for the species, but the third vial and subsequent ones were incubated again at 42°. This time the organisms grew well for 7 consecutive days at 42° and died out on the eighth transfer. The fact that the species lived for seven transfers at 42° as compared with five previously suggested some slight adaptation; but this series demonstrated again that a continuous exposure of a growing bacterial species to a temperature of 42° killed the species, although the organisms grew well for seven transfers. Again there was no gradual diminution in the turbidity of the cultures, the seventh culture incubated at 42° showing as heavy a growth as the first.

In contrast with the continuous exposure technique, we carried simultaneously the same organism for 30 consecutive daily transfers employing an alternating incubation: the odd-numbered vials in the series were incubated at 42° C. and the even-numbered vials were incubated at 37°. Without exception, vigorous growth has been obtained and the process of exposure to an alternate incubation at 42° is being continued. In fact, this organism is now able to withstand incubation at 45° for 24 hours.

### Eberthella Typhosa

*Eberthella typhosa* was grown continuously in series at 45° C., by making daily transfers of one-half milliliter of the inoculum in the same manner as described for *Streptococcus hemolyticus*.

This series displayed a good growth for 36 consecutive daily transfers at 45° C. In subsequent vials, however, the turbidity was perceptibly less after 24 hours' growth at 45°, but the normal degree of turbidity appeared at the end of the second day's incubation at 37°. The organisms died out in the forty-sixth vial, although about 1-ml. amounts had been transferred when it was noticed that the growth of the organisms during the 24 hours of incubation at 45° was slowing up. However, after all vials had aged a few days, no measurable differences in turbidity could be seen in any (Nos. 1 to 45).

A second series of *Eberthella typhosa* was carried, the alternating or *rhythmic* exposure to 45° C. being employed. The odd-numbered vials were again incubated at the higher temperature, 45°, and the even-numbered vials incubated at 37°. Vigorous growth followed for 20 days. The twenty-first vial and subsequent odd-numbered vials were incubated 24 hours at 47.5°. Again vigorous growth continued. The series, which has now reached the one hundred and forty-eighth vial was

incubated at 49° on the one hundred and thirty-first transfer. No morphologic or tinctorial changes have been observed at any time when compared with control series transferred daily and incubated at 37°.

## DISCUSSION

Observations on the effect of methylcholanthrene upon paramecia, which extended over a period of 2½ years, led to the suggestion that an environment which is apparently harmless to individual organisms for a number of successive generations, or cell-division cycles, may be finally fatal to the species. By employing two strains of bacteria, experimental proof of this idea was readily obtained in a few weeks. At the same time it was shown that if the organisms were alternately or rhythmically exposed to the same conditions throughout successive generations survival resulted rather than the death of the species.

It is our belief that these experiments throw some light on the mechanism of species adaptation to unfavorable environments. These experiments also lead us to suspect that the mechanism of carcinogenesis in mammalian fixed tissues may be basically a slow process of adjustment of the cells to a carcinogenic agent. As far as we are aware, no observations from either the clinical or experimental fields of cancer research support the idea that the genesis of cancer involves a sudden change of a normal cell. The clinically observed association of cancer with chronic irritation, the prolonged incubation period in experimentally induced cancer, and the various precancerous lesions and stages that have been described by clinicians seem to fit in with a concept of *gradualism* as a reasonable hypothesis. From this point of view, a cancer cell is a normal cell that, through chronic exposure of its progenitors to unfavorable irritating or at least unusual conditions, makes at last a successful adjustment—successful only from the standpoint of the cell itself. The adjustment is, of course, fatal from the standpoint of the organism as a whole.

It is needless to point out that when an actively growing organism is alternately exposed to temperatures far above the optimum for the species, one is setting up an artificial condition not unlike that which is known to induce cancer in human beings. We refer, of course, to the well-known skin cancer induced by the periodic use of kangri baskets in certain provinces of India.

That the genesis of cancer is primarily a process of adjustment to a variety of unfavorable environments is also suggested by the fact that the large number of physical and chemical agents known to induce a cancer is increasing rapidly.

## SUMMARY

A simple technique is described for the regular transfer of bacterial cultures which seems to reduce to a minimum the possibility of air-borne contaminations.

Results following the exposure of two species of bacteria (*Streptoccocus hemolyticus* and *Eberthella typhosa*) to high temperatures suggest that (1) a given environment harmless to the individual may be fatal to the species, provided the exposure is *continuous*, and (2) when the exposure is *rhythmic*, a species can successfully survive the same environment that is fatal when the exposure is continuous.

The role of the adjustment process as a possible mechanism in the genesis of cancer is discussed.

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# Comparative Enzymatic Activity of Transplanted Hepatomas and of Normal, Regenerating, and Fetal Liver

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## INTRODUCTION

Hepatomas may arise spontaneously or may be induced by administration of chemical carcinogens in rats and in certain strains of mice. Are the chemical properties, in particular the enzymatic properties, the same for all the hepatomas irrespective of their origin, or will they vary according to the mode of origin of the tumors or the strains in which they appear? This problem forms the basis of the present investigation.

There are available at the present time in this Institute six well-defined hepatoma transplants: (1) hepatoma 31 in rats of the Osborne-Mendel strain originally induced by the feeding of *p*-dimethylaminoazobenzene (*1*), (2) hepatoma 1 in I × dilute brown mice ($F_1$ generation) originally induced in I mice by repeated injection of 2-amino-5-azotoluene (*2*), (3) hepatoma A in C3H mice originally induced by repeated injection of 2-amino-5-azotoluene (*2*), (4) hepatoma 7A/77 in A mice originally induced by repeated injections of carbon tetrachloride (*3*), (5) hepatoma 98/15 which arose spontaneously in a C3H mouse (*4*), and (6) hepatoma 587 which arose spontaneously in an A mouse (*4*). We have, therefore, in mice, hepatomas in two different strains which arose spontaneously, hepatomas in two different strains which were induced by the same chemical carcinogen, aminoazotoluene, and two hepatomas in each of two strains, one of which was induced by a chemical carcinogen whereas the other arose spontaneously.

Several enzyme systems in these tissues were investigated, namely, arginase, catalase, xanthine dehydrogenase, amylase, thymonucleodepolymerase, and acid and alkaline phosphatase. Data on the rat hepatoma 31 and the aminoazotoluene-induced tumors in I × dilute brown and in C3H mice which had been obtained earlier have been included (*2*, *5–10*). For comparison, the data obtained earlier on normal rat and mouse livers, as well as on regenerating rat and fetal rabbit liver (*2*, *5–10*), have also been included.

## EXPERIMENTAL PROCEDURE

Hepatoma 7A/77 was in the third-, hepatoma 98/15 in the third- and fifth-, and hepatoma 587 in the second-generation transplant. The number of mice used carrying the tumors was 12 for 98/15, 6 for 587, and 6 for 7A/77. The data given on hepatomas 31, 1, and A are collected from earlier studies (*2*, *5–10*), and it need only be mentioned here that hepatoma 31 was used in the fourth-, sixth-, seventh-, twelfth-, thirteenth-, fourteenth-, and eighteenth-generation transplants, hepatoma 1 in the twenty-eighth-, forty-fifth-, forty-sixth-, forty-eighth-, and forty-ninth-generation trans-

plants, and hepatoma A in the tenth-, twelfth-, thirteenth-, and fourteenth-generation transplants. The activity of arginase in hepatoma 1 was first determined when this tumor was carried in strain I mice. Subsequently, this tumor was carried in I $\times$ dilute brown mice ($F_1$), and the activity of the other enzyme systems was determined on the tumor carried by the hybrids. Arginase (*7*), as well as catalase (*10*) studies on hepatoma 1 in the pure strain and in the hybrid mice revealed no difference in the properties of the tumor.

The preparation of the tissues for enzymatic determinations has been described, as well as the technique for each of the enzymes studied, arginase (*2*), catalase (*6, 7, 10*), xanthine dehydrogenase (*6, 7*), thymonucleodepolymerase (*5, 7, 8*), amylase (*6, 7*), and acid and alkaline phosphatase (*9*). The phosphatase activity of regenerating rat liver and the thymonucleodepolymerase activity of fetal and adult rabbit liver, not previously reported, are described in the present paper. The activity of each of the enzymes was determined for the tumors mentioned above and for the normal livers of each of the strains studied, for regenerating rat liver obtained by the excision of 60–70 percent of the normal rat liver, and for adult and fetal rabbit liver. In the case of hepatoma 7A/77, because of the small amount of material available, study of one of the enzyme systems was omitted (amylase).

Before different kinds of tissues can be compared, it is essential to know two things: (1) The reproducibility of any given determination on any one tissue; and (2) the variation of a particular determination on a given tissue from one animal to another. Unless these are known, comparative studies on a number of different tissues in various kinds of animals will have little meaning. We have found that the reproducibility of the enzymatic estimations is within 5 percent for any one tissue, and that the individual variation of a given tissue in the same group of animals was rarely greater than $\pm 20$ percent. In order to be conservative we have, therefore, considered only differences between tissues of the order of at least $\pm 50$ percent as significant.

## Catalase

The catalase activity of the various hepatomas is considerably less than that of normal liver (table 1). The activity of fetal rabbit liver is likewise much below that of normal adult rabbit liver. The activity of the normal liver in the six kinds of mice studied is the same, and that of normal and of regenerating rat liver is practically identical. The activity of hepatoma 1 in three kinds of mice, I strain, I $\times$ dilute brown ($F_1$), and I $\times$ C3H ($F_1$), is the same. The rat hepatoma is very much lower in catalase activity than any of the mouse hepatomas.

Of interest, however, is the apparent fact that the activity of the hepatomas in each strain, regardless of the origin of the tumor, is more nearly the same than is that of hepatomas in different strains which arise in apparently the same manner. Thus, the activity of the two hepatomas in the C3H strain, one of which was originally induced and the other of spontaneous origin, is of the same order. Similarly, the activity of the two hepatomas in the A strain, one of which was originally induced and the other of spontaneous origin, is also of nearly the same order. On the other hand, the activity of the hepatomas

TABLE 1.—*Catalase activity of transplanted hepatomas*

[Temperature 27°± 1° C., 1.0 cc. of 29 percent hydrogen peroxide added to mixture of 1.0 cc. extract plus 5 cc. of M/5 phosphate buffer at pH 6.9]

| Tissue | Strain | Primary tumor induced by— | Age of tumor [1] | Concentration of N in extract | Rate of oxygen evolution |
|---|---|---|---|---|---|
| | | | *Days* | *Mg. per cc.* | *Cc. per second* |
| Normal rat livers (*6*) | | | | 0.6 | 1.7 |
| Regenerating rat livers (2 days) (*6*) | | | | .3 | .5 |
| | | | | .6 | 1.7 |
| | | | | .3 | .5 |
| Transplanted hepatoma 31 in Osborne-Mendel rats (*6*). | | *p*-Dimethylaminoazobenzene. | 20–50 | 3.0 | .01 |
| | | | | 1.5 | .007 |
| Normal mice livers (*7, 10*) | A, C3H, I, dilute brown, I × dilute brown, I × C3H. | | | .6 | 2.2 |
| | | | | .3 | .8 |
| | | | | 3.0 | 2.9 |
| Transplanted hepatoma 1 (*10*) | I | 2-Amino-5-azotoluene | 21 | 1.5 | 1.7 |
| | | | | .6 | .4 |
| | | | | 3.0 | 2.9 |
| Do (*10*) | I × dilute brown | do | 21 | 1.5 | 1.5 |
| | | | | .6 | .4 |
| | | | | 3.0 | 3.0 |
| Do (*10*) | I × C3H | do | 21 | 1.5 | 1.6 |
| | | | | .6 | .4 |
| | | | | 3.0 | .3 |
| Transplanted hepatoma A (*7*) | C3H | do | 90 | 1.5 | .1 |
| | | | | 3.0 | .4 |
| Transplanted hepatoma 98/15 | C3H | Spontaneous | 60 | 1.5 | .2 |
| | | | | 3.0 | (2) |
| Transplanted hepatoma 7A/77 | A | Carbon tetrachloride | 120 | 1.5 | 3.0 |
| | | | | .6 | .8 |
| | | | | 3.0 | 1.2 |
| Transplanted hepatoma 587 | A | Spontaneous | 60 | 1.5 | .5 |
| Normal adult rabbit livers (*6*) | | | | 1.5 | 1.0 |
| Fetal rabbit livers (17 days) (*6*) | | | | 1.5 | .1 |

[1] Age of tumor refers to time elapsed since implantation.
[2] Too great to measure.

of spontaneous origin is different in C3H and in A mice, and the activity of the hepatomas originally induced by *o*-aminoazotoluene injection different in I and C3H mice. It would seem that the catalase activity of a hepatoma to at least a first approximation and in the strains investigated, is more dependent upon the strain concerned than upon the manner in which it arose.

## XANTHINE DEHYDROGENASE

Table 2 shows that activity of xanthine dehydrogenase in the rat hepatoma and the regenerating liver is less than in normal rat liver. The activity of fetal rabbit liver is much less than in normal adult rabbit liver. The activity of the normal liver in the three kinds of mice studied is the same. No change from the value of the normal livers is found in the hepatoma of I × dilute brown mice, but the values for the activity of the other hepatomas in mice are distinctly lower than the value for normal liver.

In comparing the xanthine dehydrogenase activity of the mouse hepatomas, the same picture is observed as in the case of the catalase activity. Again, the activity of the hepatomas in each strain, regardless of their origin, is more nearly alike than that of hepatomas arising in the same manner in different strains.

472192—42——2

TABLE 2.—*The xanthine dehydrogenase activity of transplanted hepatomas*

[Test: 1 cc. tissue extract plus 1 cc. M/5 phosphate buffer of pH 6.9 plus 1 cc. xanthine solution (0.0016 M) plus 1 cc. methylene blue (0.00124 M).
Control: Same as test, except 1 cc. water was substituted for the substrate. In every case more than 5 hours was required for decolorization]

| Tissue | Strain | Primary tumor induced by— | Age of tumor [1] | Concentration of N in extract | Time of decolorization |
|---|---|---|---|---|---|
| | | | *Days* | *Mg. per cc.* | *Minutes* |
| Normal rat livers (6) | | | | 2.6 | 14 |
| | | | | 1.3 | 37 |
| Regenerating rat livers (2 days) (6) | | | | 2.6 | 27 |
| | | | | 1.3 | 50 |
| Transplanted hepatoma 31 in Osborne-Mendel rats (6) | | *p*-Dimethylaminoazobenzene. | 20–50 | 2.6 | 28 |
| | | | | 1.3 | 53 |
| Normal mice livers (7) | A, C3H, I × dilute brown | | | 2.6 | 10 |
| | | | | 1.3 | 25 |
| Transplanted hepatoma 1 (7) | I × dilute brown | 2-Amino-5-azotoluene | 21 | 2.6 | 10 |
| | | | | 1.3 | 25 |
| Transplanted hepatoma A | C3H | ....do | 90 | 2.6 | 38 |
| | | | | 1.3 | 60 |
| Transplanted hepatoma 98/15 | C3H | Spontaneous | 60 | 2.6 | 35 |
| | | | | 1.3 | 70 |
| Transplanted hepatoma 7A/77 | A | Carbon tetrachloride | 120 | 2.6 | 18 |
| | | | | 1.3 | 40 |
| Transplanted hepatoma 587 | A | Spontaneous | 60 | 2.6 | 20 |
| | | | | 1.3 | 42 |
| Normal adult rabbit livers (6) | | | | 2.6 | 57 |
| Fetal rabbit livers (17 days) (6) | | | | 2.6 | >300 |

[1] Age of tumor refers to time elapsed since implantation.

## ACID AND ALKALINE PHOSPHATASE [1]

The extraordinarily high value for the alkaline phosphatase activity of the rat hepatoma 31 (table 3), which is apparently independent of the age of the tumor, has been discussed before (*9*). Not only the alkaline phosphatase but also the acid phosphatase activity of this tumor is greater than that of normal or regenerating liver. The activity of the latter two tissues is practically the same. The acid phosphatase activity of the fetal rabbit liver is higher, the alkaline phosphatase activity lower, than that, respectively, of normal adult rabbit liver.

The acid and alkaline phosphatase activity of the normal livers of the mice studied is the same. The acid phosphatase activity of the two hepatomas A and 98/15 in C3H mice and that of the two hepatomas 7A/77 and 587 in A mice have very nearly the same values. Study of the alkaline phosphatase activity, however, reveals the same picture in these tumors as was observed with catalase and xanthine dehydrogenase, for the values of this enzyme are nearly the same for the two hepatomas in C3H mice and quite different from those of the two hepatomas in A mice which also have nearly the same value. The tumors in I × dilute brown mice and in C3H mice, induced originally by *o*-aminoazotoluene injection, show nearly the same alkaline phosphatase activity (or lack of it), but different acid phosphatase activity.

*Perlmann and Ferry (11) have recently obtained evidence by fractionation experiments that the activity of acid and of alkaline phosphatase is associated with two different protein fractions of the mammalian kidney,*

TABLE 3.—*Acid and alkaline phosphatase activity of transplanted hepatomas*

[5 cc. M/300 disodium phenylphosphate incubated at pH 4.6 and 9.5 for 1 hour at 37.5° C. with 0.5 cc. extract solution]

| Tissue | Strain | Primary tumor induced by— | Age of tumor[1] | Concentration of N in extract | Hydrolysis at pH 4.6 | Q (acid) | Hydrolysis at pH 9.5 | Q (alkaline) |
|---|---|---|---|---|---|---|---|---|
| | | | *Days* | *Mg. per cc.* | *Percent* | | *Percent* | |
| Normal rat livers (9) | | | | | | 25 | | 4 |
| Regenerating rat livers (2 days) | | | | 3.0 | 100 | | 8 | 3 |
| | | | | .6 | 18 | 30 | 2 | 3 |
| | | | | .3 | 9 | 30 | 0 | |
| | | | | .15 | 4 | 27 | 0 | |
| Average | | | | | | 29 | | 3 |
| Transplanted hepatoma 31 in Osborne-Mendel rats (9) | | *p*-Dimethylamino-azobenzene. | 30 | | | 52 | | 542 |
| Normal mice livers (9) | A, C3H, I×dilute brown. | | | | | 12 | | 4 |
| Transplanted hepatoma 1 (9) | I×dilute brown | 2-Amino-5-azotoluene | 21 | | | 10 | | 1 |
| Transplanted hepatoma A (9) | C3H | do | 90 | | | 21 | | 0 |
| Transplanted hepatoma 98/15 | C3H | Spontaneous | 60 | 1.4 | 29 | 20 | 2 | 1 |
| | | | | .28 | 6 | 21 | 0 | |
| | | | | .14 | 3 | 20 | 0 | |
| | | | | .07 | 2 | 30 | 0 | |
| Average | | | | | | 22 | | 1 |
| Transplanted hepatoma 7A/77 | A | Carbon tetrachloride | 120 | 2.6 | 100 | | 7 | 3 |
| | | | | .52 | 11 | 21 | 2 | 4 |
| | | | | .26 | 6 | 23 | 1 | 4 |
| | | | | .13 | 3 | 23 | 0 | |
| Average | | | | | | 22 | | 4 |
| Transplanted hepatoma 587 | A | Spontaneous | 60 | 2.6 | 52 | 20 | 13 | 5 |
| | | | | .52 | 10 | 19 | 3 | 5 |
| | | | | .26 | 6 | 23 | 1 | 4 |
| | | | | .13 | 3 | 23 | 0 | |
| Average | | | | | | 21 | | 5 |
| Normal adult rabbit livers (9) | | | | | | 14 | | 3 |
| Fetal rabbit livers (20 days) (9) | | | | | | 22 | | 0 |

[1] Age of tumor refers to time elapsed since implantation.

With the exception of the rat hepatoma 31, all the hepatic tissues are characterized by little or no alkaline phosphatase activity. Independently of these studies and using a histologic technique with glycerophosphate as substrate, Edwards and coworkers (*1*, *3*, *4*) have observed the very high alkaline phosphatase activity in the rat hepatoma and the very slight alkaline phosphatase activity in the mouse hepatomas.

## ARGINASE

The arginase activity of the rat and of the mouse hepatomas is very much less than that of the respective normal liver of these species (table 4). Likewise the activity of fetal rabbit liver is less than that of normal adult rabbit liver. The activity of normal and of regenerating rat liver is very nearly the same. The data for the arginase activity of all of the mouse hepatomas studied reveal no differences which we would regard as significant. The activity of this enzyme appears to be very nearly the same for all of the hepatomas, regardless of strain or manner of origin.

TABLE 4.—*The arginase activity of transplanted hepatomas*

[Temperature 39° C. Mixture of 1 cc. extract, 0.4 cc. M/15 phosphate buffer at pH 8.0, 0.05 cc. of 0.2 percent manganese sulfate and 1 cc. of 0.20 percent arginine monohydrochloride incubated for 2 hours. The deproteinized filtrates were subsequently incubated with urease powder]

| Tissue | Strain | Primary tumor induced by— | Age of tumor [1] | Concentration of N in extract | Hydrolysis of arginine | Q |
|---|---|---|---|---|---|---|
| | | | *Days* | *Mg. per cc.* | *Percent* | |
| Normal rat livers (2) | | | | | | 213 |
| Regenerating rat livers (2 days) (2) | | | | | | 250 |
| Transplanted hepatoma 31 in Osborne-Mendel rats (2) | | *p*-Dimethylaminoazobenzene | 20–50 | | | 21 |
| Normal mice livers (2) | A, C3H, I × dilute brown | | | | | 260 |
| Transplanted hepatoma 1 (2) | I × dilute brown | 2-Amino-5-azotoluene | 21 | | | 40 |
| Transplanted hepatoma A (2) | C3H | do | 90 | | | 34 |
| Transplanted hepatoma 98/15 | C3H | Spontaneous | 60 | 1.4 | 42 | 36 |
| | | | | .7 | 30 | 34 |
| | | | | .35 | 24 | 33 |
| | | | | .175 | 14 | 24 |
| | | | | .088 | 10 | 22 |
| Average | | | | | | 30 |
| Transplanted hepatoma 7A 77 | A | Carbon tetrachloride | 120 | 2.2 | 50 | 37 |
| | | | | 1.1 | 40 | 38 |
| | | | | .55 | 33 | 40 |
| | | | | .28 | 24 | 36 |
| | | | | .14 | 18 | 34 |
| Average | | | | | | 37 |
| Transplanted hepatoma 587 | A | Spontaneous | 60 | 2.6 | 49 | 36 |
| | | | | 1.3 | 36 | 34 |
| | | | | .65 | 27 | 33 |
| | | | | .325 | 20 | 30 |
| | | | | .163 | 17 | 32 |
| Average | | | | | | 33 |
| Normal adult rabbit livers (2) | | | | | | 260 |
| Fetal rabbit livers (17 days) (2) | | | | | | 97 |

[1] Age of tumor refers to time elapsed since implantation.

## THYMONUCLEODEPOLYMERASE [2]

The activity of thymonucleodepolymerase (table 5) in the three kinds of rat hepatic tissues, normal liver, regenerating liver, and hepatoma 31, appears to be nearly the same. The activity of fetal and adult rabbit liver appears to be practically equal.

The activity of the two hepatomas in C3H mice is very nearly the same. This value is much greater than that observed for the originally induced tumor in I × dilute brown mice and somewhat greater than that observed for the originally spontaneous tumor and the tetrachloride-induced tumor in A mice. The difference between the activity of the *o*-aminoazotoluene-induced hepatomas in I × dilute brown and in C3H mice on the one hand and the difference between the activity of the originally spontaneous tumors in C3H and in A mice on the other, illustrate the role that strain differences may play in the chemical properties of the tumors growing in these strains.

[2] *Further evidence of the heat lability of thymonucleodepolymerase was obtained by heating an aqueous mouse-liver extract which contained 1.23 mg. N per cubic centimeter in a boiling water bath for 10 minutes. Under these conditions only a trace of protein coagulated and separated from the solution. After cooling and mixing with an equal volume of thymonucleate* (5), *no evidence of enzymatic depolymerization was observed.*

TABLE 5.—*The thymonucleodepolymerase activity of transplanted hepatomas*[1]

| Tissue | Strain | Primary tumor induced by— | Age of tumor[2] | Concentration of N in extract | Activity |
|---|---|---|---|---|---|
| | | | *Days* | *Mg. per cc.* | |
| Normal rat livers (*8*) | | | | 1.23 | 6.61 |
| Regenerating rat livers (2 days) (*8*) | | | | 1.23 | 6.26 |
| Transplanted hepatoma 31 in Osborne-Mendel rats (*8*) | | *p*-Dimethylaminoazobenzene | 20–50 | 1.23 | 5.26 |
| Normal mice livers (*8*) | A, C3H, I × dilute brown | | | 1.23 | 14.32 |
| Transplanted hepatoma 1 (*8*) | I × dilute brown | 2-Amino-5-azotoluene | 21 | 1.23 | 11.07 |
| Transplanted hepatoma A (*8*) | C3H | do | 90 | 1.23 | 21.32 |
| Transplanted hepatoma 98/15 | C3H | Spontaneous | 60 | 1.23 | 20.62 |
| Transplanted hepatoma 587 | A | do | 60 | 1.23 | 15.16 |
| Transplanted hepatoma 7A/77 | A | Carbon tetrachloride | 60 | 1.23 | 15.42 |
| Normal adult rabbit livers | | | | 1.23 | 10.46 |
| Fetal rabbit livers (17 days) | | | | 1.23 | 10.42 |

[1] According to method described (*8*).
[2] Age of tumor refers to time elapsed since implantation.

## AMYLASE [3]

The activity of amylase (table 6) is the same for all the rat hepatic tissues studied, normal liver, regenerating liver, and hepatoma 31, even though glycogen is nearly completely lacking from the last-mentioned tissue (*1*).

Comparison of the activity of this enzyme in the mouse hepatomas studied reveals that the activity for each tumor is apparently different from that of each of the other tumors. This presents a situation not hitherto encountered among the other enzyme systems studied in the mouse hepatomas, where the enzymatic activity was either very nearly the same for all the tumors or else showed differences based on the particular strains used (tables 1–5). Of all the mouse hepatomas only that carried in the I × dilute brown mice has the same activity as normal mouse liver. Glycogen is present in all of the mouse hepatomas (*1*, *3*, *4*).

[3] *The enzymatic reaction to which we give the name amylase and which is followed by the appearance of oxidizable groups is probably due to at least two separate enzymes* (12).

TABLE 6.—*The amylase activity of transplanted hepatomas*

[Incubation temperature 39° C. Data in the last column represent difference in titer of the mixture with and without added glycogen minus the value due to the original reducing groups of the glycogen]

| Tissue | Strain | Primary tumor induced by— | Age of tumor[1] | Concentration of N in extract | Time of incubation | 0.02 N thiosulfate |
|---|---|---|---|---|---|---|
| | | | *Days* | *Mg. per cc.* | *Hours* | *Cc.* |
| Normal rat livers (*6*) | | | | 2.6 | 1 | 0.7 |
| | | | | | 2 | 1.5 |
| | | | | | 4 | 2.3 |
| | | | | 1.3 | 1 | .5 |
| | | | | | 2 | .9 |
| | | | | | 4 | 1.5 |
| Regenerating rat livers (2 days) (*6*) | | | | 2.6 | 1 | .8 |
| | | | | | 2 | 1.6 |
| | | | | | 4 | 2.3 |
| | | | | 1.3 | 1 | .4 |
| | | | | | 2 | .9 |
| | | | | | 4 | .15 |

[1] Age of tumor refers to time elapsed since implantation.

TABLE 6.—*The amylase activity of transplanted hepatomas*—Continued

| Tissue | Strain | Primary tumor induced by— | Age of tumor | Concentration of N in extract | Time of incubation | 0.02 N Thiosulfate |
|---|---|---|---|---|---|---|
| | | | *Days* | *Mg. per cc.* | *Hours* | *Cc.* |
| Transplanted hepatoma 31 in Osborne-Mendel rats (6). | | *p*-Dimethylaminoazobenzene. | 30 | 2.6 | 1 | 1.0 |
| | | | | | 2 | 1.5 |
| | | | | | 4 | 1.8 |
| | | | | 1.3 | 1 | .5 |
| | | | | | 2 | .9 |
| | | | | | 4 | 1.8 |
| Normal mice liver (7) | A, C3H, I × dilute brown. | | | 2.6 | 1 | 1.6 |
| | | | | | 2 | 2.9 |
| | | | | | 4 | 4.1 |
| | | | | 1.3 | 1 | 1.3 |
| | | | | | 2 | 2.1 |
| | | | | | 4 | 3.2 |
| Transplanted hepatoma 1 (7) | I × dilute brown | 2-Amino-5-azotoluene | 21 | 2.6 | 1 | 1.7 |
| | | | | | 2 | 2.8 |
| | | | | | 4 | 4.0 |
| | | | | 1.3 | 1 | 1.2 |
| | | | | | 2 | 2.1 |
| | | | | | 4 | 3.1 |
| Transplanted hepatoma A (7) | C3H | do | 90 | 2.6 | 1 | 3.1 |
| | | | | | 2 | 4.0 |
| | | | | | 4 | 4.6 |
| | | | | 1.3 | 1 | 2.4 |
| | | | | | 2 | 3.0 |
| | | | | | 4 | 3.9 |
| Transplanted hepatoma 98/15 | C3H | Spontaneous | 60 | 2.6 | 1 | 2.0 |
| | | | | | 2 | 3.0 |
| | | | | | 4 | 4.0 |
| | | | | 1.3 | 1 | 1.6 |
| | | | | | 2 | 2.4 |
| | | | | | 4 | 3.0 |
| Transplanted hepatoma 587 | A | do | 60 | 2.6 | 1 | 1.3 |
| | | | | | 2 | 2.4 |
| | | | | | 4 | 3.4 |
| | | | | 1.3 | 1 | 1.0 |
| | | | | | 2 | 1.4 |
| | | | | | 4 | 2.4 |
| Normal adult rabbit livers (6) | | | | 2.6 | 1 | .7 |
| | | | | | 2 | .9 |
| Fetal rabbit livers (17 days) (6) | | | | 2.6 | 1 | .5 |
| | | | | | 2 | .8 |

## DISCUSSION

It has been one of the purposes of the present series of studies on the several enzymatic properties of tumors to make comparisons with those of the normal tissue of origin of the tumors (*2*, *5–10*). The availability of a number of well-defined hepatomas in various strains and kinds of laboratory animals has permitted the extension of such comparisons to studies between the possible varieties of the same kind of tumor (tables 1–6). To this may be added some information on spontaneous mammary tumors in different strains of mice (*2*, *7*, *9*). When these comparisons are made, it is obvious that no simple generalization which covers the behavior of all or even of any one group of tumors emerges from the data obtained at the present time. Such statements as that of Edlbacher, that all tumors are characterized by a higher arginase content than normal tissues, have certainly been based on inadequate material and faulty comparison (*2*, *13*, *14*).

The details of the comparison between tumors and the normal tissues from which they arose and of the comparison between tumors of a single group of tissues have been given in earlier papers in this series of studies (*2*, *5–10*) and in the section, Experimental Procedure, in the present paper. Briefly stated, the former comparison reveals that when a normal tissue becomes neoplastic certain enzyme systems may decrease in activity, others may increase, and still others may not change.

The direction and order of magnitude of the change may depend on the species of animal, the strain in any given species as well as the kind of tumor under consideration. Thus, the enzymes, arginase and catalase, are uniformly lower in activity in all hepatomas as compared with normal liver; but in mammary tumors the arginase activity is greater than, and the catalase activity equal to that of normal mammary tissues. Again, the arginase activity of mouse lymphomas is greater than, the catalase activity less than that, respectively, of normal mouse lymph nodes. For these enzymes, the kind of tissue is decisive in the changes involved. The acid and alkaline phosphatase activity of the rat hepatoma is higher than that of normal liver. The acid phosphatase activity of the mouse hepatomas is either equal to or greater than, whereas the alkaline phosphatase activity is either equal to or less than, the corresponding activity in normal mouse liver. For these enzymes, the change in activity when the normal liver becomes tumorous will depend not only on the species of animal but also on the strain of mouse in which the tumor occurs. The same conclusion may be reached from the data on thymonucleodepolymerase, amylase, and xanthine dehydrogenase.

Substantially the same picture is obtained from independent sources. Thus, it has been stated that the activity of the following systems is lower in hepatomas than in normal liver, transaminase (*15*), cytochrome *c* (*16*), succinate oxidation (*17*), and riboflavin (*18*). On the other hand, Maver, Mider, Johnson, and Thompson (*19*) have reported that the proteinase activity of the rat hepatoma is greater than that of normal liver.

The second comparison, that between several tumors arising differently in different species and strains from the same kind of tissue, such as the hepatomas (tables 1–6), has revealed that these hepatomas in mice may for certain enzyme systems be equal in activity, for other enzyme systems be equal in each strain but differ from strain to strain, and for other systems differ distinctly from tumor to tumor. Thus, arginase belongs to the first category; catalase, xanthine dehydrogenase, thymonucleodepolymerase, and to a certain extent acid and alkaline phosphatase, to the second category; and amylase to the third category. These relations hold for mouse hepatomas. For mammary tumors in mice, not only arginase but also catalase and the phosphatases belong to the first category. For the lymphomas in mice, arginase belongs to the third category.

It is clear by now that any discussion of the enzymatic activity of tumors in comparison with that of normal tissues or of other tumors must take into account the following factors: (1) The particular enzyme system considered; (2) the kind of tumor; (3) the species of animal; (4) the strain of animal in which the tumor occurs; and (5) the corresponding value of enzymatic activity in the normal tissue from which the tumor arose. As a rule, therefore, each tumor must be considered on its own merits. As far as we have been able to observe, from the quantitative study of the several enzyme systems and the many tumors with which we have been concerned, there does not appear to be a generalization which will cover all phases of tumor enzymology.

## SUMMARY

The activity of arginase, catalase, xanthine dehydrogenase, amylase, thymonucleodepolymerase, and acid and alkaline phosphatase was determined in the following transplanted hepatomas: 98/15 in C3H mice which arose spontaneously, 7A/77 in A mice which arose by repeated

injection of carbon tetrachloride, and 587 in A mice which arose spontaneously in this strain. Comparison of the data was made with data obtained earlier on hepatoma 1 in I mice and hepatoma A in C3H mice, both of which had arisen in these strains by repeated injection of *o*-aminoazotoluene. Further comparison was made with the enzymatic data obtained before with transplants of hepatoma 31 in rats, originally induced by the feeding of *p*-dimethylaminoazobenzene, with fetal rabbit liver, regenerating rat liver, and with the normal liver of all the strains and species of animals studied. Discussion based on these comparisons was supplemented by observations on the enzymatic activity of normal and cancerous mammary and lymphatic tissues.

Comparison between tumors and the normal tissues from which they arose reveals that when a normal tissue becomes neoplastic certain enzyme systems may increase, others decrease, and others remain unchanged in activity. The direction of the change, if any, may depend upon the species of animal, the strain of mouse, as well as the kind of tumor under consideration. Comparison between the hepatomas arising differently in various strains of mice reveals that the activity of certain enzyme systems may be the same for all the tumors, that the activity of certain systems may be equal for hepatomas of different origin in the same strain but may differ from strain to strain, and that the activity of still other enzyme systems may differ distinctly from tumor to tumor.

The conclusion is reached that the enzymology of each tumor must be considered on its own merits and that the following factors must be taken into account: (1) The particular enzyme system considered; (2) the kind of tumor; (3) the species of animal; (4) the strain of animal; and (5) the activity of the normal tissue of origin. At the present time there does not appear to be a generalization which will cover all phases of the quantitative enzymatic activity of tumors.

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# Induction of Cirrhosis of the Liver and of Hepatomas in Mice With Carbon Tetrachloride

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A report on the induction of hepatomas in strains A and C3H mice by repeated oral administration of carbon tetrachloride, together with a brief description of the tumor, appeared in an earlier paper (*1*). Subsequently hepatomas have been induced in other strains of mice, and additional data have been accumulated on dosage. In this paper our observations on carbon tetrachloride administration to mice are brought up to date, and a more detailed account is given of the pathologic changes observed.

## EXPERIMENT 1. INCIDENCE OF SPONTANEOUS HEPATOMAS IN UNTREATED CONTROL MICE

The mice employed were all virgin. They included strain C3H males of the Andervont line, males and females of strain A and of strain Y, and strain C females.[1]

The animals were fed Purina dog chow pellets from weaning and had access to an unlimited supply of water. Autopsy following cervical dislocation was performed when the animals were of varying ages. In this way a number of the animals were examined at the age of 1 year or less and so were directly comparable with the experimental group, but many were studied when well over a year of age. While the latter were older than the animals in the experimental groups which follow, it seems justifiable to include the figures on these since the incidence of spontaneous hepatomas in a group of older mice is higher than in younger mice of respective strains. Such a difference serves only to make the figures on induced tumors less striking.

Many of these data have been taken from the published and unpublished work of Andervont (*2*). The results are summarized in table 1. Male C3H mice of the Andervont line 11–19 months of age showed an incidence of 25.8 percent of spontaneous hepatomas. The figures broken down indicate that at 11 months the incidence was 10 percent and rose from this value to a peak of 50 percent at 15 months of age. In C3H mice under 11 months of age, hepatomas were extremely rare.

TABLE 1.—*Incidence of spontaneous hepatomas in untreated mice*

| Strain | Sex | Mice autopsied | Age of mice at autopsy | Incidence of hepatomas |
|---|---|---|---|---|
| | | *Number* | *Months* | *Percent* |
| C3H | M | 50 | 8–11 | 4.0 |
| C3H | M | 320 | 12–19 | 26.9 |
| Y | M+F | 129 | 10–16 | 1.6 |
| C | F | 150 | 13–24 | .0 |
| A | M+F | 400 | 4–8 | .0 |
| A | M+F | 400 | 12–16 | 1.5 |

The incidence of spontaneous hepatomas in the mice of strains Y, C, and A remained under 2 percent even when the animals were well over a year of age. Among 800 male and female strain A mice from

[1] *The strain A and strain Y mice were a few generations removed from the Roscoe B. Jackson Memorial Laboratory colonies of the respective strains. The strain C mice originally obtained from the Jackson Laboratory in the $F_{35}$ generation were bred in our laboratory by Dr. H. B. Andervont and examined from that generation and subsequent ones including the $F_{45}$.*

4 to 16 months of age the incidence of hepatoma was 0.75 percent. The six tumors of the liver found in this group were in mice 12 months old or over. In 129 strain Y mice of both sexes from 10 to 16 months of age 2 hepatomas were found, an incidence of 1.55 percent. No hepatomas were observed in 150 female strain C mice from 13 to 24 months of age.

## EXPERIMENT 2. REPEATED ORAL ADMINISTRATION OF CARBON TETRACHLORIDE IN THE DOSE OF 0.04 CC.[2]

It was determined that a dose of 0.1 cc. of a 40-percent solution of carbon tetrachloride in olive oil given by stomach tube [3] to adult mice caused hepatic necrosis. This dose of 0.04 cc. of carbon tetrachloride, while definitely toxic to the liver, failed to cause renal tubular necrosis and was not lethal on repeated administration. The experimental animals, when 1 to 5 months of age were started on 3 treatments weekly or occasionally only 2. The number of treatments varied from 23 to 58, but a number of other animals were examined post mortem after they had received from 1 to 23 doses in order to study the early pathologic changes. The animals were fed Purina dog chow and allowed a free supply of water.

Tumors were not found in animals which received less than 23 doses. In the group of 263 mice of strains C3H, A, Y, and C, 1 year of age or less at autopsy, which had received from 23 to 58 doses of 0.04 cc. of carbon tetrachloride, the incidence of hepatoma was 88.2 percent.[4] The percentages of hepatoma incidence for the individual strains are given in table 2.

TABLE 2.—*Incidence of hepatomas in mice given 23 to to 58 doses of 0.04 cc. of carbon tetrachloride*

| Strain | Sex | Mice autopsied | Age of mice at autopsy | Incidence of hepatomas |
|---|---|---|---|---|
| | | *Number* | *Months* | *Percent* |
| C3H | M | 143 | 6–10 | 88.1 |
| Y | M+F | 15 | 4–12 | 60.0 |
| C | F | 41 | 6–7 | 82.9 |
| A | M+F | 64 | 4–12 | 98.4 |
| Total | | 263 | Average | 88.2 |

### PATHOLOGY

#### *Liver*

The livers of mice receiving continuous treatment eventually showed cirrhosis. Within 24 hours after the first dose, there was extensive necrosis of the liver involving the central one-third to one-half of the lobule[5] (fig. 1, *A*). The cytoplasm of the parenchymal cells was swollen and eosinophilic, and there was loss of nuclei. Necrosis of sinusoidal endothelium was not apparent. There were numerous mitotic figures in the viable peripheral portion of the lobule even in this early stage, and regeneration was evident through the entire period of carbon tetrachloride administration. While many of the mitoses appeared normal, atypical forms such as triple mitoses were frequent (fig. 2, *A*). In addition, the viable zones often exhibited huge uninucleated giant parenchymal cells (fig. 2, *B*). The central necrotic cells progressively became fragmented, phagocytosed, and for the greater part removed (fig. 1, *B*).

[2] *The carbon tetrachloride used in all the experiments here reported was purchased from the Day Chemical Co., Newark, N. J. Samples examined by Dr. J. S. L. Hartwell, of our laboratory, were reported to be an excellent grade of carbon tetrachloride, containing no appreciable amounts of phosgene, other halogenated hydrocarbons, or sulfur compounds.*

[3] *The stomach tube used in these experiments was devised by Morris and Thompson* (3).

[4] *All the C3H mice used in the experiments reported in this paper were of the Andervont line. The Y mice were from Dr. Andervont's colony, and all the strain A and C mice in this and the following experiments were obtained from the Roscoe B. Jackson Memorial Laboratory.*

[5] *Unless otherwise indicated, all the photomicrographs in this paper were made from sections stained with haematoxylin and eosin.*

In about 2 weeks the centers of the lobules were largely cleared of necrotic material leaving only the supporting connective tissue and wide sinusoids with as yet no extensive replacement by regenerated cells (fig. 3, *A*).

Occasionally clumps of cellular debris became calcified and remained in the liver. These clumps were usually surrounded or engulfed by multinucleated cells which resembled foreign-body giant cells (fig. 4, *A*). As a rule a moderate amount of collagen

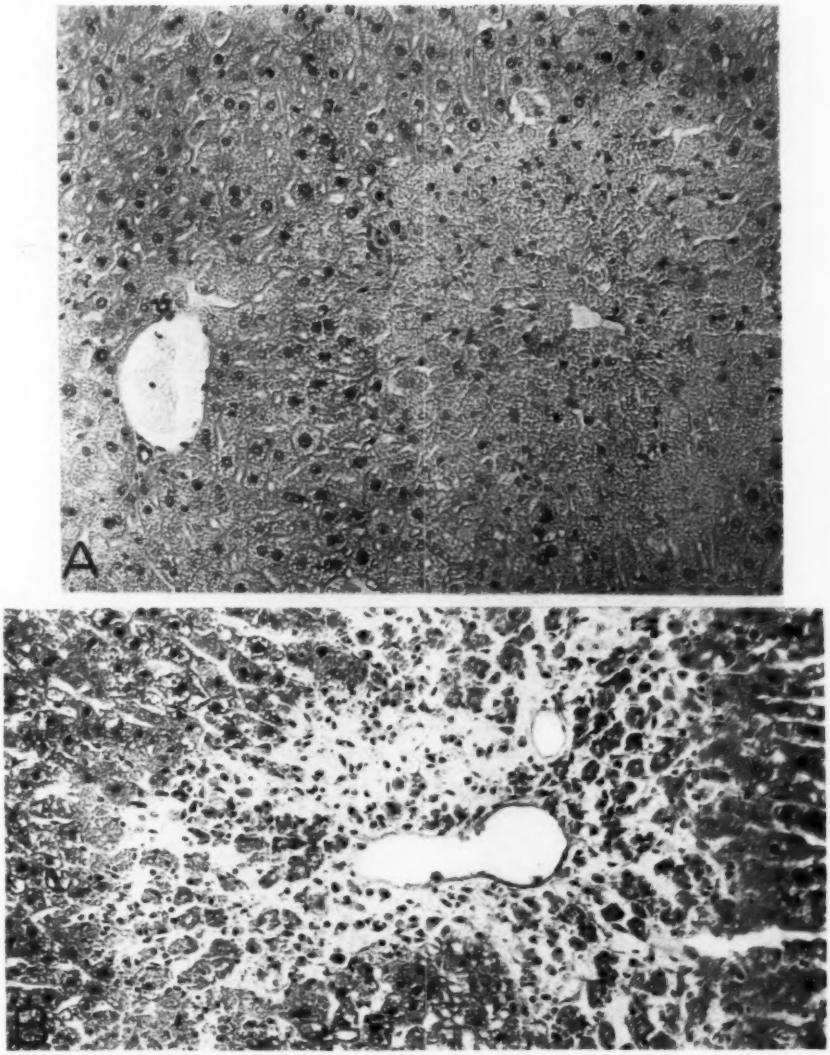


FIGURE 1.—Livers of carbon tetrachloride-treated mice: *A*, Early central necrosis with loss of parenchymal cell nuclei. ×160; *B*, Partial removal of necrotic cells in center of lobule. ×160.

or reticulum became concentrated about foci. In a mouse of strain Y there was ossification in one of these foci (fig. 4, *B*).

With repeated administration of carbon tetrachloride the central zones showed some increase in connective tissue, together with progressive restoration by regenerated parenchyma (fig. 3, *B*). Coincidentally there was extension of the necrotizing process through the lobule

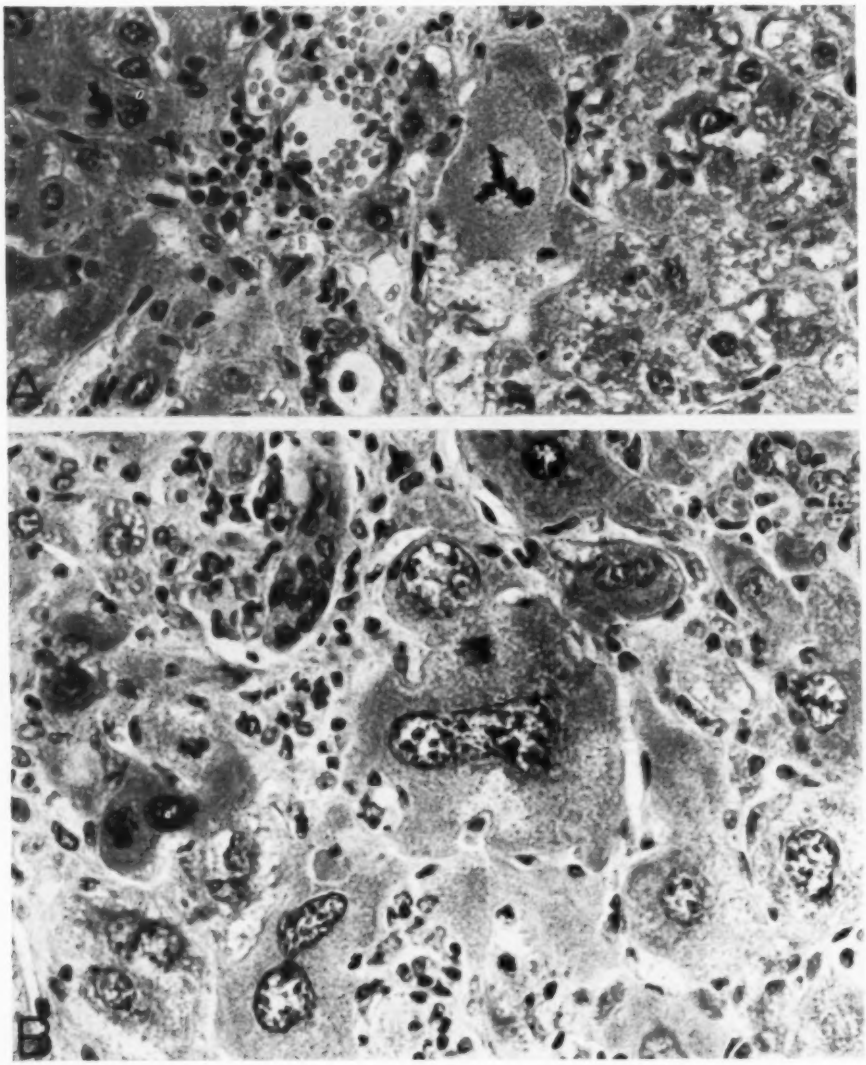

FIGURE 2.—Atypical cells in nontumorous portions of liver following repeated administration of carbon tetrachloride: *A*, Triple mitosis. ×510; *B*, Giant parenchymal cell. ×495.

to the portal zone. Ultimately the architecture of the liver was greatly disturbed. There was an increase in connective tissue distributed mainly in the vicinity of the portal areas but also extending away from these zones in an irregular fashion setting

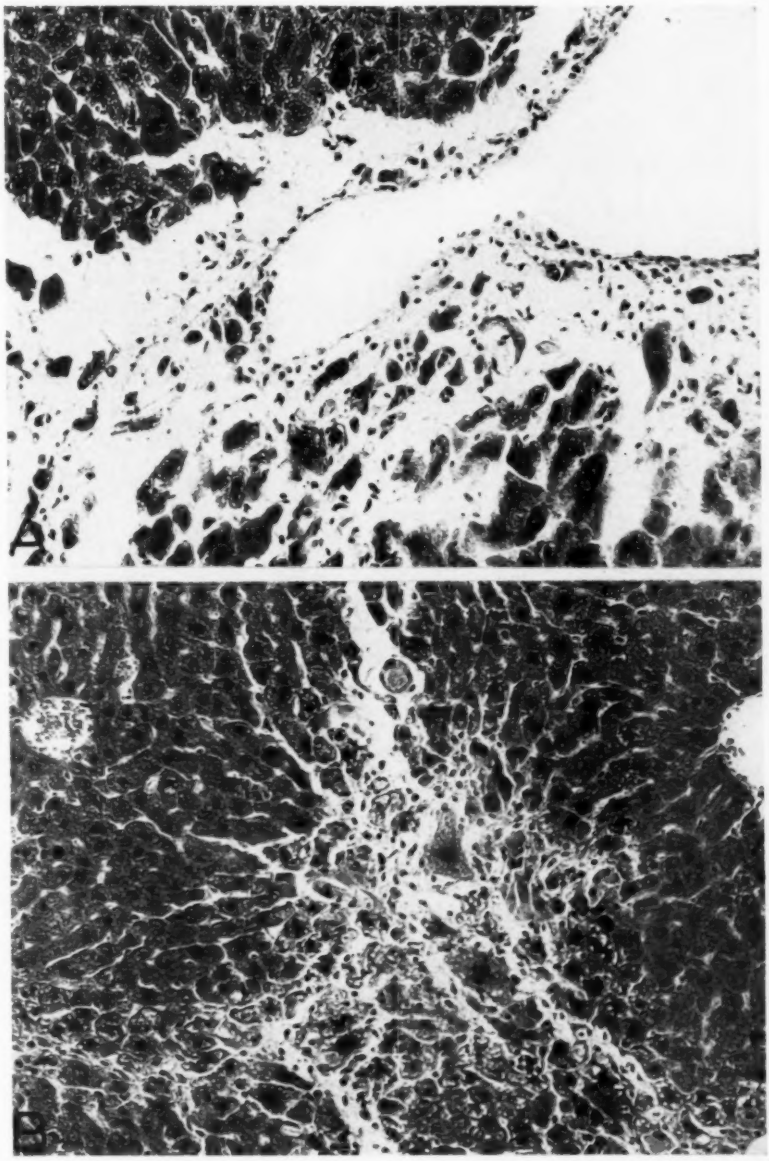

FIGURE 3.—Livers of carbon tetrachloride-treated mice: *A*, Almost complete removal of central necrotic cells. ×160; *B*, Partial regeneration. ×160.

off the parenchyma into lobules of irregular size and shape (fig. 5, *A* and *B*). Proliferation of bile ducts, while occasionally seen, was the exception rather than the rule.

A granular dark-brown pigment and a

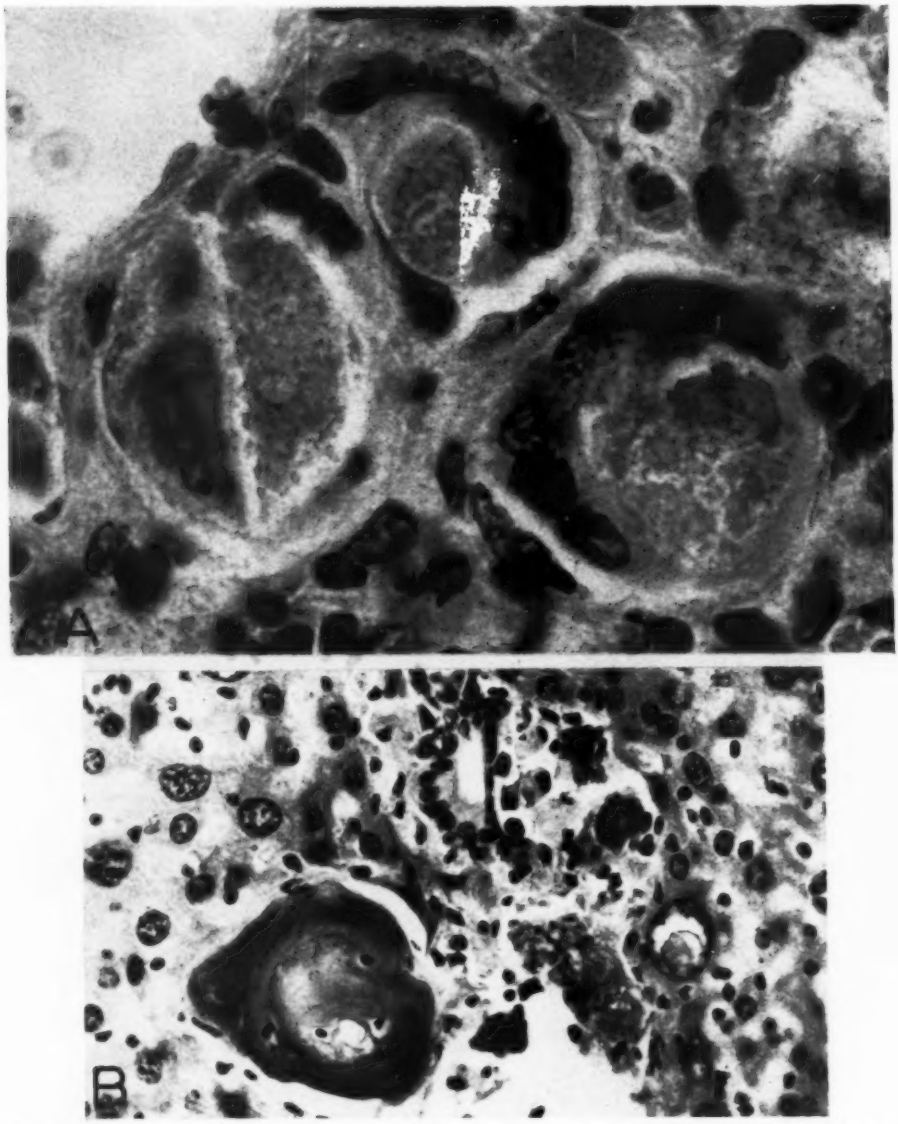


FIGURE 4.—*A*, Calcified debris in center of lobule surrounded or engulfed by cells resembling foreign-body cells. ×1360; *B*, Focus of bone probably resulting from ossification of debris similar to that seen in *A*. ×450.

diffuse canary-yellow pigment were observed in Kupffer cells and in macrophages lying within areas of fibrosis (fig. 6, *A*). The pigments gave a negative reaction for iron and did not change color with the haematoxylin and eosin stain or with dilute basic fuchsin. With Verhoeff's and the Ziehl-Neelsen stain for acid-fast bacilli, applied to paraffin sections, the brown granular pigment remained un-

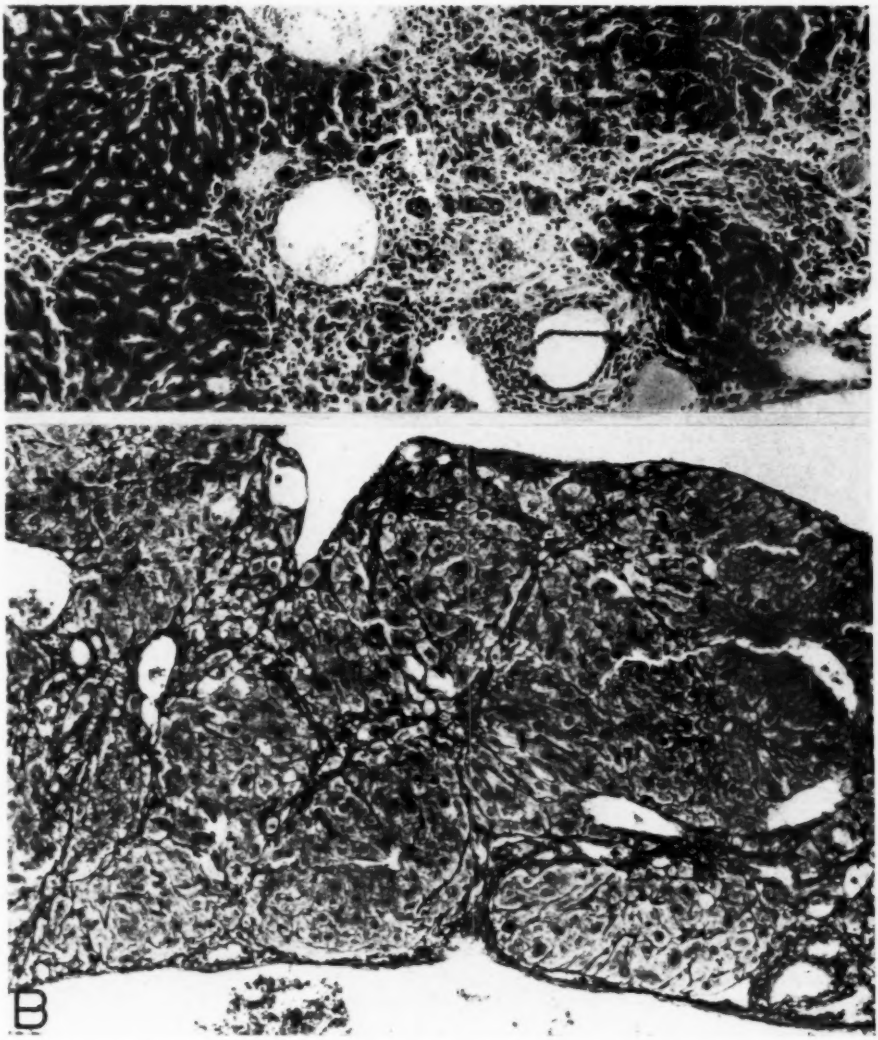


FIGURE 5.—*A*, Fibrosis of liver involving central as well as portal areas following repeated carbon tetrachloride treatment. × 140; *B*, Fibrosis and distortion of architecture characteristic of cirrhosis. Laidlaw's stain for reticulum. × 125.

changed, but the canary-yellow pigment showed a strongly positive acid-fast reaction. In addition this latter pigment gave a positive reaction for lipoids with osmic acid and sudan IV in paraffin sections.

Often in the livers of mice that had been receiving carbon tetrachloride for 1 month or over, large cells with a peculiar faintly basophilic reticulated cytoplasm and small nuclei made their appearance. These

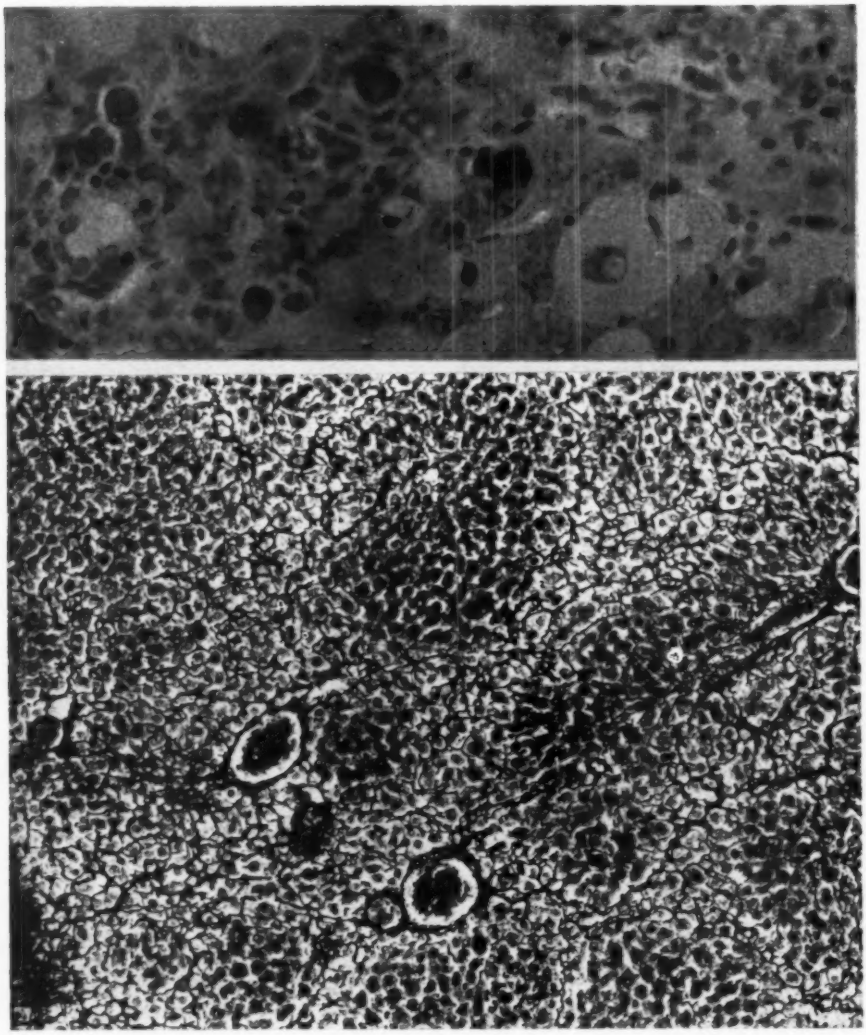

FIGURE 6.—*A*, Pigmented macrophages in an area of hepatic fibrosis. Large cells with clear, reticulated cytoplasm beside fibrous tissue. Verhoeff's acid-fast stain. × 500; *B*, Increase in reticulum in a liver 6 months after the last of 36 treatments with doses of 0.04 cc. carbon tetrachloride. Laidlaw's stain for reticulum. × 125.

were observed in varying numbers and tended to show a particular concentration along strands of fibrous tissue (fig. 6, *A*).

One series of strain A mice received 36 feedings of carbon tetrachloride followed by a period of some weeks or months during which no treatment was given. In these mice the livers at autopsy showed considerable restoration of parenchyma. So apparently nearly normal were the haematoxylin and eosin preparations that it was often difficult to be certain that any lesion was present aside from the presence of the canary-yellow pigment described above. However, with Mallory's aniline blue connective tissue stain or its Lee-Brown modification and with Laidlaw's method for demonstrating reticulum, livers of these animals showed an appreciable increase in connective tissue and reticulum. This increase was regularly demonstrated in the portal areas, extending spiderlike into the lobule and occasionally involving the middle and central zones as well (fig. 6, *B*). Occasionally such livers showed foci of calcified debris associated with foreign-body giant cells as described previously.

### *Primary Tumors*

The hepatic tumors observed in this experiment were usually multiple, as many as 10 occurring in 1 liver. They varied from 0.1 to 2.0 cm. in diameter, and a rough correlation was observed between the size of the tumor and duration of the experiment. The smaller tumors were reddish gray and bulged above the hepatic capsule. The larger were soft, either gray or yellow, and a few of these were pedunculated and hung suspended from the liver by a pedicle of shrunken hepatic tissue.

Microscopically, the tumors were non-encapsulated, well-differentiated hepatomas compressing the adjacent hepatic tissue which often showed a local increase in reticulum (fig. 7, *A*). The tumor consisted of cords, often one or two cells thick, alternating with endothelial-lined sinuses (fig. 7, *B*). The tumor cells had prominent cell margins; granular, occasionally vacuolated, cytoplasm; and relatively large vesicular nuclei with one to three prominent basophilic nucleoli (fig. 8, *A*). They resembled hepatic parenchymal cells from which they could usually be distinguished by their faintly basophilic cytoplasm. The hepatoma cell showed considerable variation in size in some tumors. In many it was larger, and in a few it was smaller than the normal hepatic cell. Occasional giant cells were observed. Mitotic figures varied considerably in number, and some were atypical (fig. 8, *B*). A number of tumor cells contained varying sized spherical cytoplasmic inclusions that usually stained red with haematoxylin and eosin, occasionally blue or faintly green. No intracellular pigment was seen in these tumors. The connective tissue of the tumors was usually scanty, appearing as interrupted strands of reticulum between the sinusoid and the epithelial cord. In a few tumors the connective tissue was more abundant lying interposed between the epithelium and narrowed sinusoid (figs. 7, *A*, and 9, *A*). At times the cords of tumor cells radiated from a centrally placed vessel, suggesting a lobular arrangement. This feature was not constant, however, and portal canals were absent. Well-differentiated bile ducts occupying no regular position were found in a number of the tumors. Most often these structures were found toward the periphery of the growth, suggesting that they were included by extension of the tumor into adjacent hepatic tissue rather than representing an integral part of the tumor. No invasion of blood vessels by hepatoma was seen, even though rather wide blood vessels were often in contact with the periphery

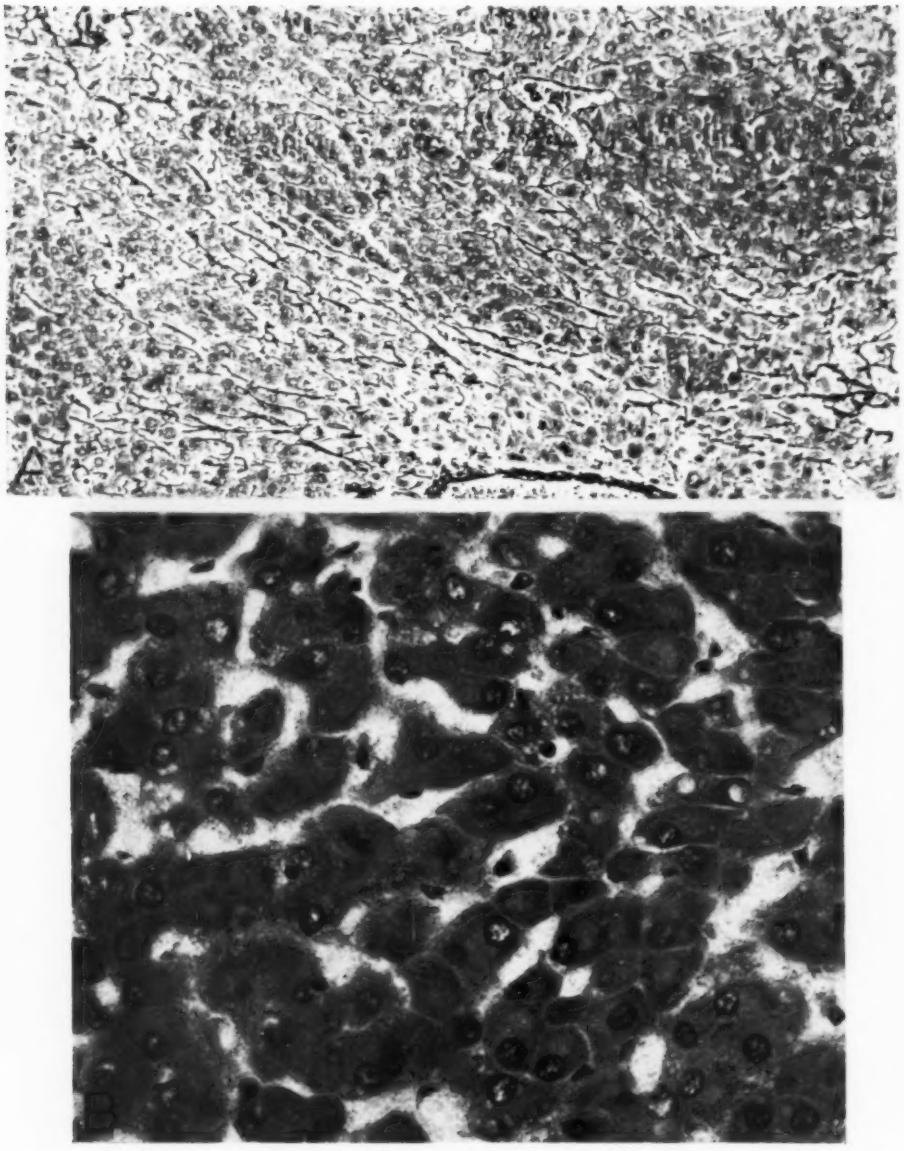

FIGURE 7.—*A*, Hepatoma and adjacent hepatic tissue. Reticulum fibers along cords of tumor cells. Increase in reticulum in hepatic tissue compressed by tumor. Laidlaw's stain for reticulum. ×138; *B*, Carbon tetrachloride-induced hepatoma. ×500. See transplant of this tumor in figure 10.

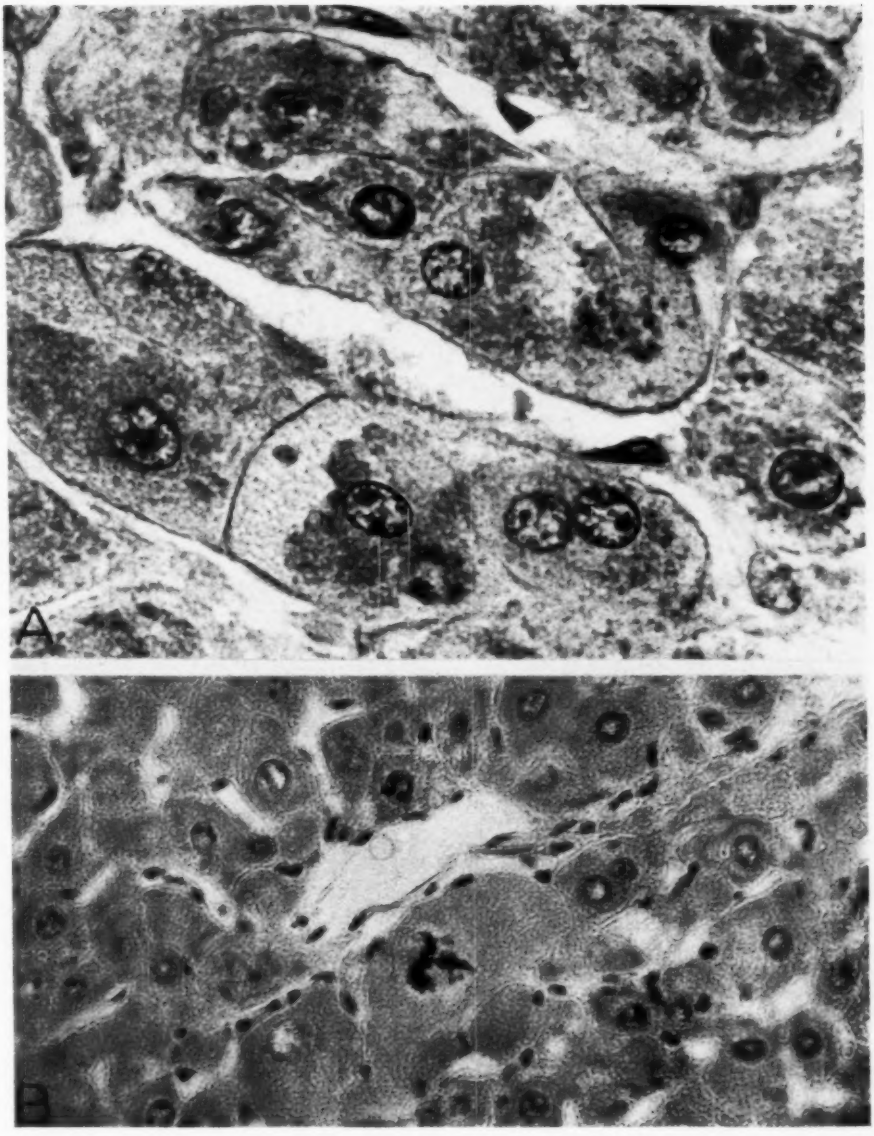


FIGURE 8.—*A*, Carbon tetrachloride-induced hepatoma. ×1120; *B*, Triple mitosis in hepatoma. ×510.

of the tumor nodules (fig. 9, *B*). Metastases were not observed. No adenocarcinomas or hemangio-endotheliomas were present in any of the livers. No tumors appeared to have been induced in any of the other organs.

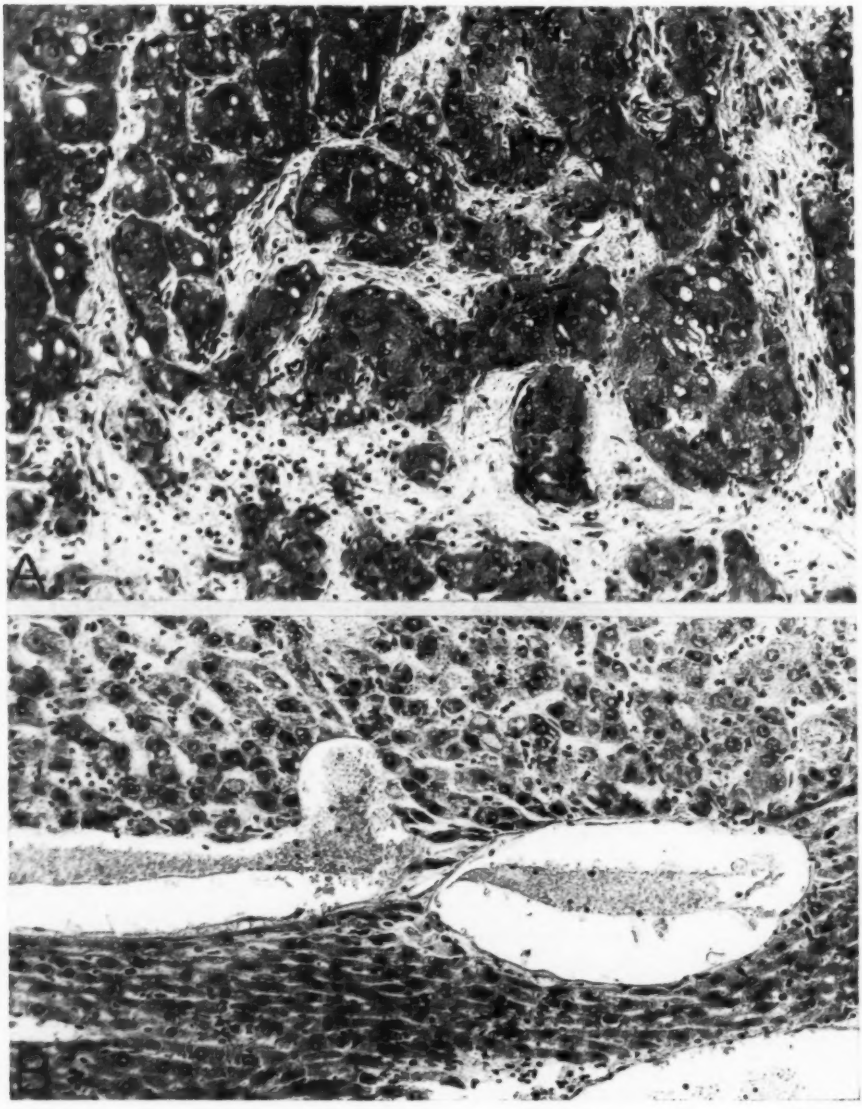


FIGURE 9.—*A*, Primary hepatoma showing considerable increase in connective tissue. ×183; *B*, Hepatoma closely approximated to blood vessels without invading these. ×165.

### Transplantation

Subcutaneous transplants of tumor tissue were made by the trocar technique into mice of homologous strains. Of eight tumors transplanted, four arose in strain C3H and four in strain A mice. Only one tumor was successfully transplanted, that originating in a strain A male mouse. The surviving recipient strain C3H animals are still under observation after a period of 4 to 15 months. The strain A recipients still without tumor received transplants 10 months ago.

The tumor (7A/77) yielding successful transplants arose in a strain A male mouse that had been started on doses of 0.04 cc. of carbon tetrachloride at 2 months of age. After 36 doses the drug was discontinued, and the animal was allowed to live for an additional 6 months. At autopsy the liver showed several soft gray and yellowish-gray hepatomas averaging 1.0 cm. in diameter.

Pieces of one tumor were transplanted to four strain A mice, and the remainder was fixed separately. Of the four animals inoculated, two showed transplants 1.0 cm. in diameter 3 months later. The tumor has been carried from these two transplant lines in strain A mice and is now in the fourth generation. Microscopic examination of the primary hepatoma yielding these transplants revealed a well-differentiated hepatoma with only a moderate number of mitoses (fig. 7, *B*). There was no evidence of unusual rate of growth, the tumor appearing very similar to those in which transplantation has not as yet been successful. The subcutaneous transplants were invasive and have continued to exhibit a structure similar to that of the primary growth (fig. 10). No pigment other than occasional hemosiderin granules in phagocytes has been demonstrated.

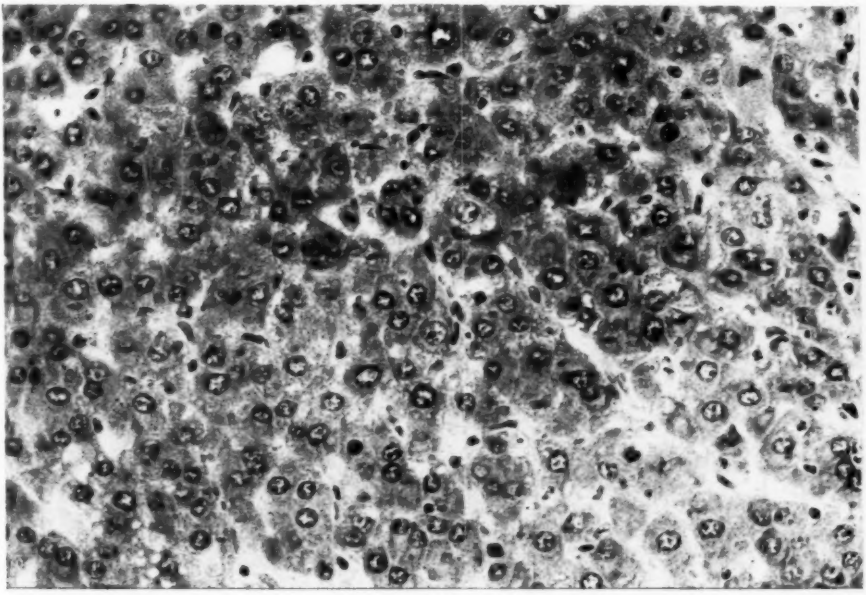

FIGURE 10.—Transplanted carbon tetrachloride-induced hepatoma. Second generation. ×330. See primary tumor, figure 7, *B*.

### Special Techniques

Several special microscopic techniques have been applied to a number of primary tumors and transplants. These include the sudan IV and osmic acid stain for fat, Best's carmine stain for glycogen, Gomori's method for demonstrating alkaline phosphatase (*4*), a modified Regaud's method for mitochondria, and Nassanow's modification of Kalatchew's method for demonstrating the Golgi apparatus (*5*).

Fat occurred abundantly as moderately large droplets in the cytoplasm of the primary and transplanted tumor cells. Glycogen was likewise present in considerable amounts. Tests for alkaline phosphatase in primary neoplasms revealed little or none within the cytoplasm of the tumor cells.

The mitochondria present in the cells of both primary and transplanted carbon tetrachloride-induced hepatomas were predominantly fine tenuous filaments (fig. 11, *A* and *B*) as described previously (*5*). The mitochondrial pattern was found similar to that of the cells of *o*-aminoazotoluene-induced hepatomas of the mouse but different from that of spontaneous hepatomas in which the mitochondria were predominantly medium-sized spheres. Each of these three types of hepatomas could be distinguished from normal hepatic tissue on the basis of the mitochondrial pattern since in the latter a characteristic intralobular variation from spherical to filamentous forms exists.

The Golgi apparatus of the cells of carbon tetrachloride-induced hepatomas was found to be basically similar in form and position to that of spontaneous and *o*-aminoazotoluene-induced hepatomas. It assumed a characteristic juxtanuclear position (fig. 12, *A*) in contrast with its partially peripheral distribution in normal hepatic cells (*5*). While basically similar to that of other hepatomas, the Golgi apparatus differed in detail. In cells of carbon tetrachloride-induced hepatomas it was characteristically greatly hypertrophied, and this condition persisted in transplants (fig. 12, *B*).

## EXPERIMENT 3. INCIDENCE OF HEPATOMAS IN MICE RECEIVING OLIVE OIL REPEATEDLY

Eighty-three male mice of strains C3H, C, and A were given 0.1 cc. of olive oil repeatedly 2 or 3 times weekly for 39 to 62 doses. The olive oil was from the same lot as that used in experiment 2. Autopsy was performed when the mice were from 5 to 12 months of age. The results are summarized in table 3. Only 1 hepatoma was found in a strain C3H male mouse 11 months of age. This is in keeping with the expected incidence of spontaneous hepatoma, and the solitary character of the tumor encountered is likewise consistent with a spontaneous rather than an induced neoplasm. Other than this tumor, no lesion was found in any of the livers. Stains for reticulum revealed no increase in this substance and no disturbance of architecture (fig. 13). A small amount of canary-yellow pigment was present in a rare Kupffer cell.

Table 3.—*Incidence of hepatoma in mice given 39 to 62 doses of 0.1 cc. of olive oil*

| Strain | Sex | Mice autopsied | Age of mice at autopsy | Incidence of hepatoma |
|---|---|---|---|---|
| | | *Number* | *Months* | *Percent* |
| C3H | M | 23 | 10-11 | 4.3 |
| C | M | 16 | 12 | .0 |
| A | M | 44 | 5-12 | .0 |

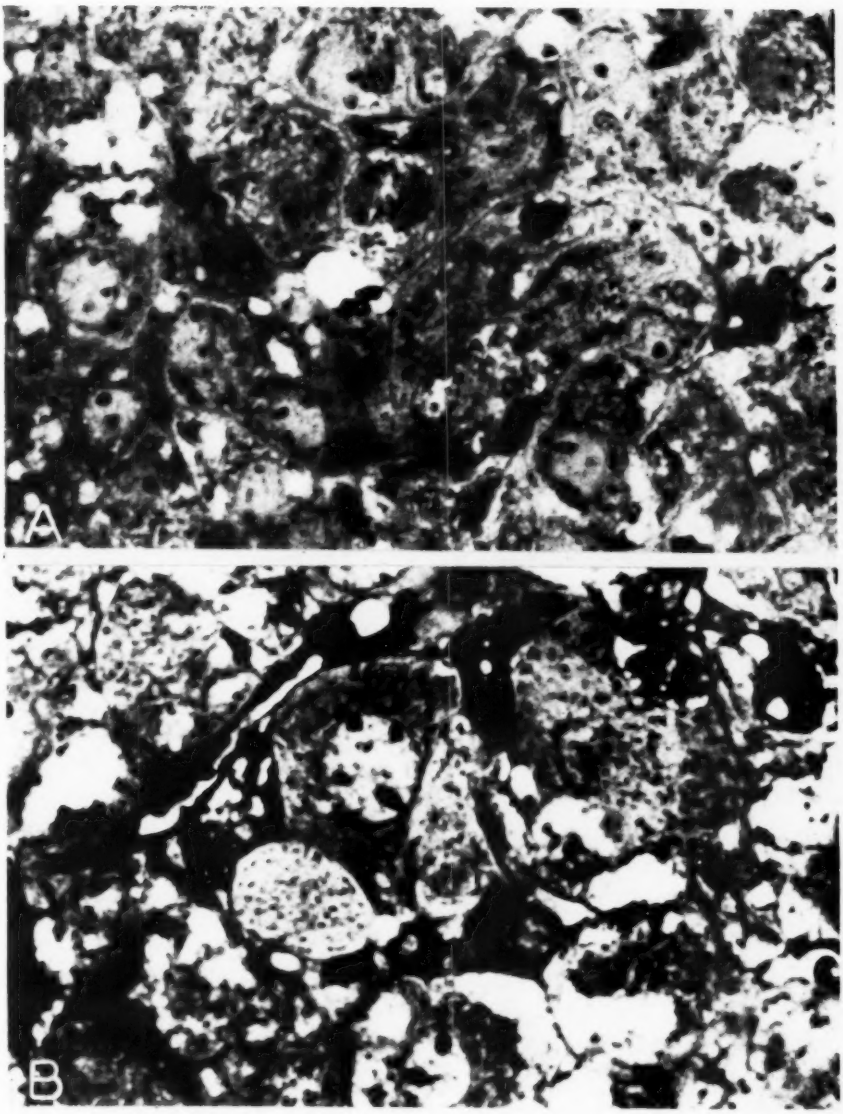


FIGURE 11.—Predominately filamentous mitochondria in carbon tetrachloride-induced hepatoma. Regaud fixation. Iron haematoxylin stain. × 1300. *A*, Primary hepatoma; *B*, Transplanted hepatoma, third generation.

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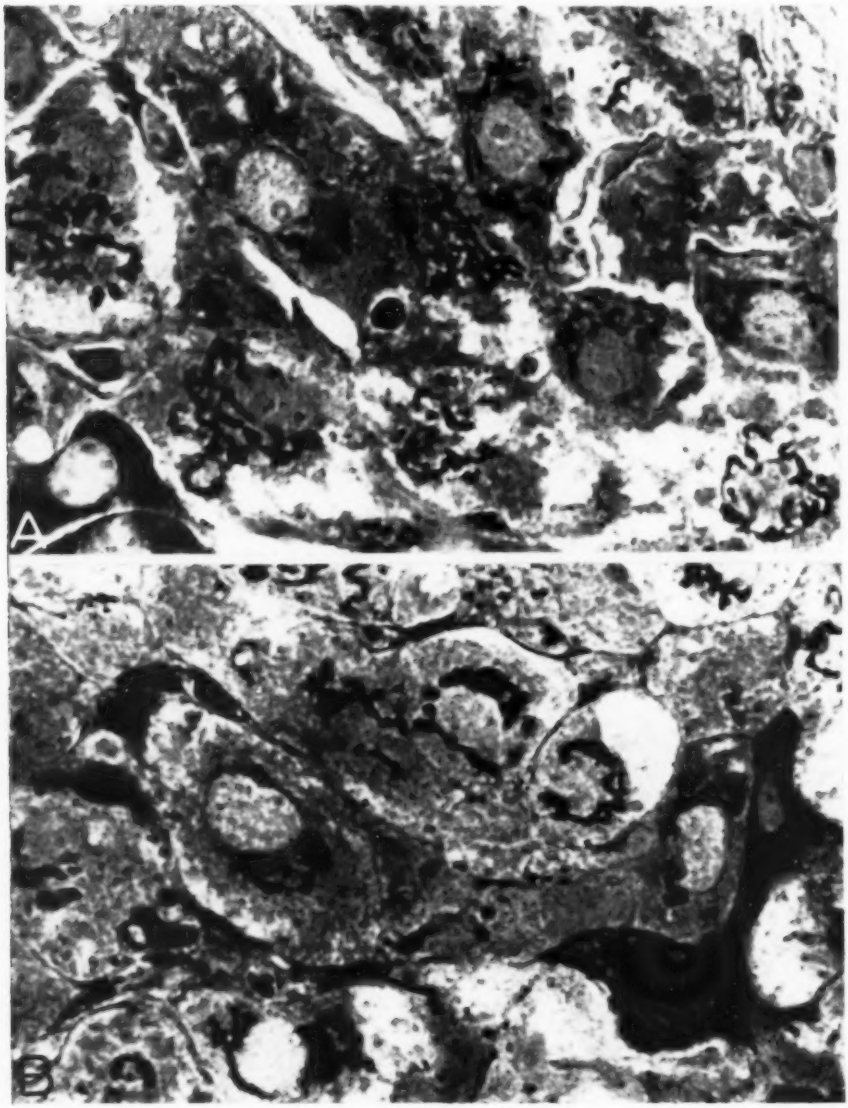



FIGURE 12.—Golgi apparatus in carbon tetrachloride-induced hepatomas. Juxtanuclear hypertrophied network. Osmic acid impregnation. × 1300. *A*, Primary hepatoma; *B*, Transplanted hepatoma, third generation.

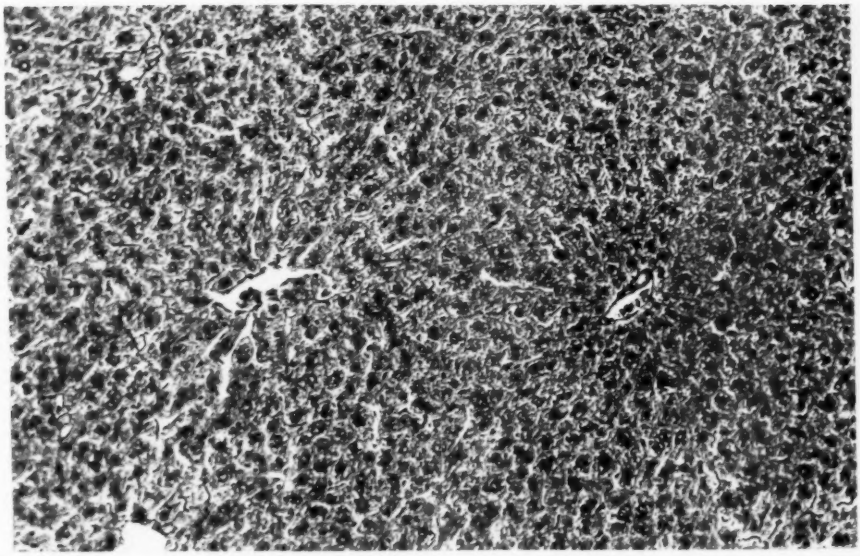


FIGURE 13.—Normal reticulum distribution in liver after repeated oral administration of olive oil. × 140

## EXPERIMENT 4. EFFECT OF LIMITED TREATMENT WITH CARBON TETRACHLORIDE ADMINISTERED ORALLY

Strain A female mice, 2 months of age, were used in experiment 4. The carbon tetrachloride used was dissolved in olive oil, the volume of the mixture administered amounting to 0.1 cc. All of these dosages were hepatotoxic. Eleven mice received only 0.1 cc. of olive oil by stomach tube. The animals were autopsied from 2 to 12 months after the start of the experiment when they were from 4 to 14 months of age. The results are given in table 4.

TABLE 4.—*Incidence of hepatomas in female strain A mice receiving small doses of carbon tetrachloride and of olive oil*

| Dose (in cubic centimeters) | Number of treatments | Number of mice | Age of mice at autopsy | Incidence of hepatomas |
|---|---|---|---|---|
| Carbon tetrachloride: | | | *Months* | *Percent* |
| 0.04 | 1–3 | 62 | 4–14 | 0 |
| 0.01 | 1 | 18 | 9–12 | 0 |
| 0.005 | 1 | 21 | 12 | 0 |
| Olive oil: | | | | |
| 0.1 | 1 | 11 | 12 | 0 |

No tumors were found in any of the animals. The livers showed complete regeneration. The only evidence of earlier damage was a moderate number of Kupffer cells containing acid-fast canary-yellow pigment, occasional foci of basophilic debris surrounded by foreign-body giant cells in the central zones (fig. 14, *A*), and an increase in connective tissue and reticulum which was readily evident with special stains (fig. 14, *B*). The increase in reticulum was usually identified centrally where single parenchymal cells were often encircled by these fibers (fig. 14, *B*).

## EXPERIMENT 5. REPEATED ORAL ADMINISTRATION OF CARBON TETRACHLORIDE IN THE DOSE OF 0.005 CC.

One-tenth of a cubic centimeter of 5 percent carbon tetrachloride in olive oil

was administered 3 times weekly to 58 female strain A mice 2.5 months old for from 25 to 29 stomach-tube feedings. Thus, in this experiment each mouse received a total of from 0.125 to 0.145 cc. of carbon tetrachloride over a period of about 2 months. This dosage is quite comparable with the total dose of 0.120 cc. of carbon tetrachloride administered to those animals of experiment 4 which received 3 treatments of 0.04 cc. each. Autopsies performed from 48 hours to 4.5 months after the last feeding showed hepatomas in 41 mice, an incidence of

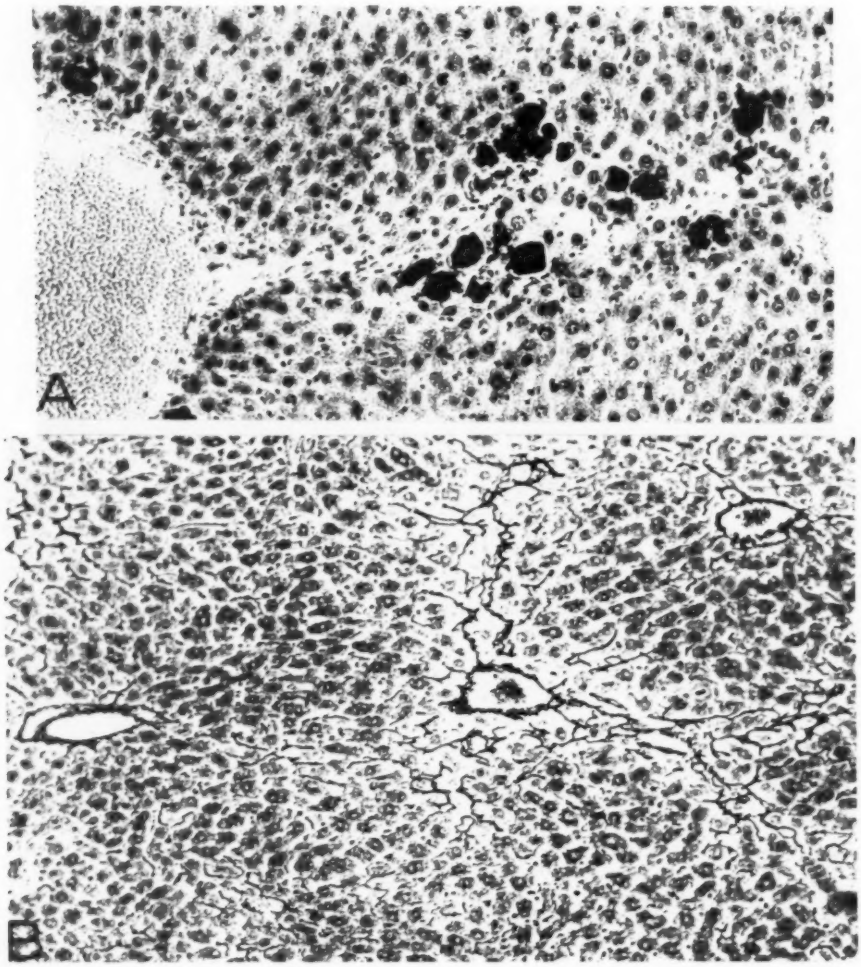



FIGURE 14.—*A*, Foci of calcified debris in liver 10 months after one treatment with 0.04 cc. carbon tetrachloride. ×193; *B*, Increase in intralobular reticulum, mainly central, 10 months after one treatment with 0.005 cc. carbon tetrachloride. Laidlaw's stain for reticulum. ×147.

70.7 percent. The tumors and the pathologic changes in the livers were similar to those seen in the animals of experiment 2, which had received 0.04 cc. of carbon tetrachloride repeatedly. Those animals autopsied shortly after the last treatment showed a definite distortion of hepatic architecture and fibrosis characteristic of cirrhosis. Those animals that were autopsied 1 month or more after the last dose presented considerable restoration of parenchyma with the presence of pigmented macrophages. With stains for reticulum there was demonstrated a distortion of lobular architecture and an increase in reticulum along the cords and about the blood vessels (fig. 15).

## DISCUSSION

The nodules of liverlike tissue arising in the livers of mice receiving the hepatotoxic substance, carbon tetrachloride, were studied with the object of determining whether they are regenerative or neoplastic in nature. The liverlike structure of these growths, together with the capacity of their epithelial cells to store fat and glycogen, does not rule out neoplasm, because these features are found in primary and transplanted spontaneous and *o*-aminoazotoluene-induced hepatomas in the mouse which are considered true neoplasms (*6*, *7*).

Other features the carbon tetrachloride-induced hepatoma has in common with

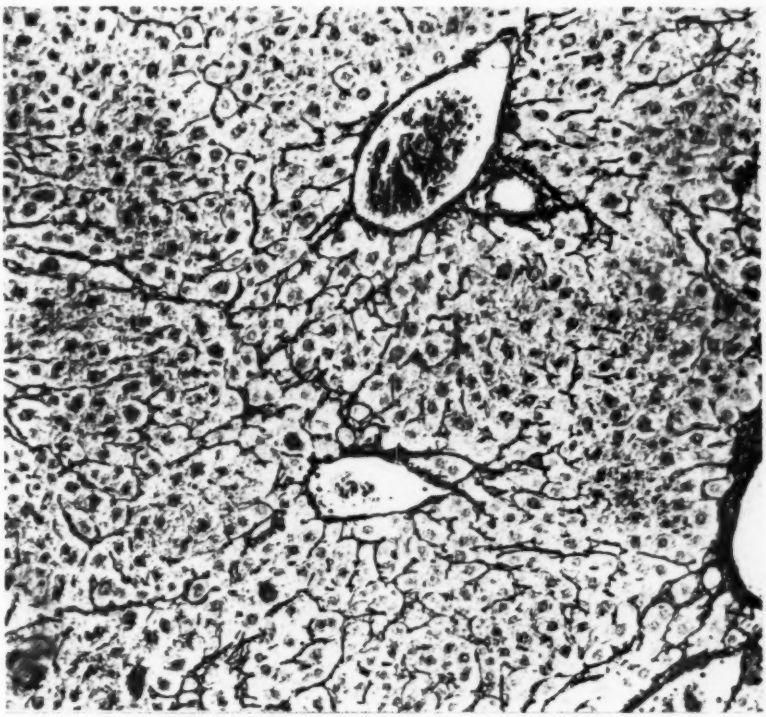


FIGURE 15.—Intralobular increase in reticulum throughout hepatic lobule 2 months after the last of 29 treatments of 0.005 cc. of carbon tetrachloride. ×147.

these latter tumors which serve to distinguish them all as a group from regenerating tissue are the frequent basophilic character of the cytoplasm of the epithelium as opposed to the usual eosinophilic cytoplasm of normal and regenerating hepatic cells, transplantability (*7, 8, 9*), progressive growth, and a specific type of Golgi apparatus (*5, 7*) which is essentially similar to that of primary and transplanted *p* - dimethylaminoazobenzene - induced hepatomas of the rat (*10, 11*). The mitochondria of the carbon tetrachloride-induced hepatomas are similar to those of *o*-aminoazotoluene-induced hepatomas in the mouse (*5*) and the *p*-dimethylaminoazobenzene-induced hepatomas in the rat (*10, 11*) but differ from those of the spontaneous hepatoma (*5, 7*) and of normal and regenerating liver in the mouse (*5*). In addition to these morphologic considerations, the carbon tetrachloride-induced hepatoma has a resemblance chemically to other hepatomas in the mouse.

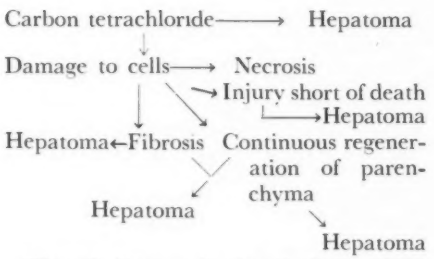
Robertson and Kahler (*12*) observed that the riboflavin content of the transplanted carbon tetrachloride-induced hepatoma was $56.9 \pm 16.4$ micrograms per gram of dry weight of tissue. This value is essentially similar to that of $47.1 \pm 13.0$ micrograms per gram of dry weight for transplanted *o*-aminoazotoluene-induced hepatomas in the mouse, and $51.4 \pm 12.7$ micrograms of riboflavin per gram of dry weight for the transplanted spontaneous hepatoma in the same species. These values are distinctly below those for nontumorous livers in this animal. Robertson [6] has studied the riboflavin content of the normal liver, of hepatic tissue 48 hours after partial hepatectomy, and of liver 48 hours after the last of three hepatotoxic doses of a carbon tetrachloride-olive oil solution (0.04 cc. carbon tetrachloride) administered every other day by stomach tube. The number of micrograms of riboflavin per gram of dry weight in this series was higher than in the hepatomas, being $97 \pm 3.0$, $101.5 \pm 7.5$, and $96.0 \pm 5.6$, respectively.

The absence of stainable alkaline phosphatase from carbon tetrachloride-induced hepatomas parallels our findings in the case of the transplanted spontaneous hepatoma (*7*) and is consistent with those of Greenstein who did quantitative studies for this enzyme on transplants of the carbon tetrachloride-induced hepatoma, and the *o*-aminoazotoluene-induced, and spontaneous hepatomas in the mouse (*13*). These low values for alkaline phosphatase appear to be universal in the case of mouse hepatomas, regardless of how they are induced. In contrast, the *p*-dimethylaminoazobenzene-induced hepatomas of the rat show high values for this enzyme by both quantitative and staining techniques (*10, 13*). This suggests that the difference depends upon species.

In addition to the foregoing work on the comparative chemistry of the carbon tetrachloride-induced hepatoma, there is the report of Greenstein and coworkers (*14*) in which are recorded quantitative studies for a number of enzymes on hepatomas and normal tissues. In the case of a number of enzymes, notably arginase, catalase, and alkaline and acid phosphatase, the values for the mouse carbon tetrachloride-induced hepatoma correspond to those for spontaneous and *o*-aminoazotoluene-induced hepatomas in the same species.

While the mechanism of tumor genesis by repeated administration of carbon tetrachloride remains obscure, the pathologic picture of the liver suggests several theoretical explanations which may serve as avenues of approach in investigating this problem. They are summarized in the following diagram:

[6] *Unpublished work.*




Though interest in the cirrhosis which developed in the mice of our experiments is secondary to that of tumor formation, there are several features of this lesion which should be mentioned.

As far as we are able to determine, earlier experiments with carbon tetrachloride in mice have been only of short duration, directed chiefly at toxicity studies with no description of the effects of long-continued administration (*15*, *16*). In the case of other species, notably the rat (*17*), pig (*18*), and dog (*19*), however, a number of authors have reported induction of carbon tetrachloride cirrhosis. In those animals, as in the mouse, the early lesion was central necrosis of the hepatic lobule. With continuous administration of the compound, fibrosis involving the periphery of the lobule developed.

In order for cirrhosis to occur, it was necessary to treat the mice for about 6 weeks. This phenomenon has likewise been described for animals of other species in which a small number of treatments with the drug is reported to have failed to produce fibrosis. Moreover, it was stated that continuous administration of the compound was necessary for the evident cirrhosis to persist (*17*).

In our animals a single hepatotoxic dose gave rise to an increase of reticulum within the center of the lobule which was evident many months after administration of the carbon tetrachloride. It is interesting to study the changes following the withdrawal of carbon tetrachloride from mice that had received the drug for several months. Those animals which were autopsied shortly after the last treatment showed cirrhoses. If, however, autopsy was performed a month or more after the last treatment, the picture was different. With the haematoxylin and eosin stain such livers often showed very little evidence of previous damage, but the reticulum preparations revealed distortion of lobules and an increase in reticulum.

An increase in connective tissue may be of considerable importance functionally in cirrhoses, perhaps of as much significance as regeneration. Claims that completely normal livers result when animals have been allowed a period of rest after carbon tetrachloride treatment should be accepted with some reserve, particularly if no attention has been paid to the amount of fine connective tissue or reticulum present.

The canary-yellow pigment observed in the livers of our mice appears to be a lipoid, on the basis of its staining reactions with Verhoeff's and Ziehl-Neelsen's stains for acid-fast bacilli, osmic acid, and with sudan IV. Its lack of solubility in organic solvents such as alcohol, cedar oil, or xylol, through which the tissues passed during the processes of paraffin embedding and staining would indicate that this substance is not purely lipoidal in nature. The explanation that it is a lipoid conjugated with some other molecule (*20*), as, for example, a protein, appears compatible with both its staining and solubility reactions. These reactions of this pigment are the same as those given by a canary-yellow pigment which occurs in the livers of rats on a basal diet of low protein and high fat supplemented with *p*-dimethylaminoazobenzene, as described by Edwards and White in 1941 (*20*, *21*). Following the reports of these authors, Lillie and his coworkers (*22*) described similar

staining reactions for a pigment which was present in the livers of rats maintained on a low protein diet. While in the case of the dietary experiments on the rat, it might be suggested that this pigment is the product of abnormal metabolism, its presence in our mice, which were on a presumably well balanced diet but which had extensive hepatic necrosis, might indicate that the pigment is derived from the products of disintegrated parenchymal cells.

## SUMMARY AND CONCLUSIONS

Repeated oral administration of carbon tetrachloride in doses of 0.04 cc. in olive oil to 263 mice of strains C3H, A, C, and Y, which were autopsied when they were 1 year of age or less, resulted in a hepatoma incidence of 88.2 percent. This figure is distinctly above the incidence of spontaneous hepatomas in these strains.

The tumors were well-differentiated hepatomas and resembled the spontaneous and *o*-aminoazotoluene-induced tumors of the mouse. Both the mitochondrial pattern and the Golgi apparatus of carbon tetrachloride-induced hepatomas were similar to those of other hepatomas in the mouse and rat. One tumor of eight in which transplantation was attempted is transplantable.

The benignancy or malignancy of the carbon tetrahcloride-induced hepatoma in the mouse has not as yet been established.

Repeated oral administrations of olive oil failed to raise the incidence of hepatoma above that for the spontaneous variety. It is, therefore, felt that in the work with the carbon tetrachloride-olive oil mixture the carbon tetrachloride was the hepatoma-producing agent.

Single or limited doses of carbon tetrachloride in concentrations sufficient to cause hepatic necrosis failed to induce tumors. However, if the same total amount of carbon tetrachloride was given in divided doses over a period of 2 months, hepatomas developed in 71 percent of the mice.

Repeated administration of hepatotoxic doses of carbon tetrachloride results in cirrhosis of the liver within a period of about 6 weeks. Removal of the compound after this lesion has developed results in considerable restoration of parenchyma after a month's time. Fibrosis becomes less evident, but there is a permanent increase in intralobular reticulum. Single doses of carbon tetrachloride likewise result in a permanent increase in intralobular reticulum.

In the livers of carbon tetrachloride-treated mice there is an abundance of a canary-yellow pigment which, while insoluble in organic solvents, gives a positive reaction for lipoids with sudan IV and osmic acid, and is acid-fast with Verhoeff's and with the Ziehl-Neelsen stain for acid-fast bacilli. This material is, perhaps, a conjugated lipoid.

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# Effect of Supplementary Methionine or Choline Plus Cystine on the Incidence of *p*-Dimethylaminoazobenzene-induced Hepatic Tumors in the Rat

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Recent observations have indicated that the occurrence of fatty infiltration as well as cirrhosis of the liver is markedly influenced by certain dietary constituents. Best, Hershey, and Huntsman (*1*) produced fatty livers in rats and were able to prevent this lesion by feeding purified lecithin or one of the components of lecithin, namely, choline. Beeston and Channon (*2*) reported that the addition of cystine to a diet low in choline produced a marked increase in liver fat and that the cystine effect could be offset by giving additional casein. Tucker and Eckstein (*3*) found that methionine had a marked lipotropic effect. These authors concluded that the methionine in casein was responsible for the lipotropic effect. Du Vigneaud et al. (*4, 5*) demonstrated by using deuteromethionine that methionine can furnish the methyl group for the synthesis of choline in animals on a choline-free diet. Earle and Victor (*6*) produced cirrhosis in rats by the oral administration of a diet low in protein with 5–10 percent added cystine. György and Goldblatt (*7, 8*) reported that rats on a low casein-high fat diet exhibited hepatic injury between 100 and 150 days and that the addition of *l*-cystine produced an enhancing effect on the production of cirrhosis of the liver. The addition of choline instead of cystine to this low casein-high fat diet reduced the severity and the incidence of hepatic injury. These investigators further reported that the combined administration of *l*-cystine plus choline or *dl*-methionine was highly effective in preventing liver injury. Blumberg and McCollum (*9*) also found that choline is effective in preventing cirrhosis in rats on high fat-low protein diets. Lowry, Daft, Sebrell, and Lillie (*10*) produced cirrhosis on a low choline-low casein diet to which cystine had been added. A marked improvement in the gross appearance of the liver and hyperplastic regeneration of liver cells was observed following treatment with choline, a high casein diet, or choline with a high casein diet. György, Poling, and Goldblatt (*11*) reported the effect of choline and cystine in the production of hepatic tumors in rats fed a diet containing *p*-dimethylaminoazobenzene. They conclude—

> that the lipotropic activity of casein and in particular the combined oral administration of cystine plus choline afford a definite but not regular protection against pathological changes in the liver (necrosis, cirrhosis, atypical nodular proliferation of the bile ducts, adenocarcinoma, malignant hepatoma) produced by a diet containing dimethylaminoazobenzene.

It has been suggested that the production of hepatic tumors by the oral administration of *p*-dimethylaminoazobenzene to rats is somewhat dependent on the development of cirrhosis as an initial step. If this be true, then the prevention or marked reduction of the degree of cirrhosis should

TABLE 1.—*Effect of dietary supplements on the production of hepatic tumors by* p-*dimethylaminoazobenzene*

| Group No. | Diet | Number of animals | Number of tumor-bearing animals | Time | Tumor development |
|---|---|---|---|---|---|
| | | | | *Months* | *Percent* |
| 1 | Basal | 30 | 0 | 4–16 | 0 |
| 2 | Basal+0.5 percent *l*-cystine | 30 | 0 | 5–14 | 0 |
| 3 | Basal+0.06 percent *p*-dimethylaminoazobenzene+0.5 percent *l*-cystine+0.2 percent choline | 30 | 27 | 3–8 | 90.0 |
| 4 | Basal+0.06 percent *p*-dimethylaminoazobenzene+0.5 percent methionine | 20 | 18 | 5–11 | 90 |
| 5 | Basal+0.06 percent *p*-dimethylaminozaobenzene | 80 | 48 | 4–16 | 60.0 |
| 6 | Basal+0.06 percent *p*-dimethylaminoazobenzene+0.5 percent *l*-cystine | 87 | 84 | 4–16 | 96.5 |

lead to a reduction or retardation in the incidence of induced hepatic tumors produced by the oral administration of *p*-dimethylaminoazobenzene. We previously reported (*12*) the effect of added cystine on the incidence of *p*-dimethylaminoazobenzene-induced hepatic tumors. The results of these experiments are included in table 1.

## EXPERIMENTAL PROCEDURE AND RESULTS

Male Osborne-Mendel rats individually housed at weaning were placed on the diet of White and Jackson (*13*). The salt mixture used was the modified Osborne-Mendel mixture (*14*). When the animals reached a weight of about 80 gm., they were divided into four groups: Group 1 was placed on the basal diet; group 2 received the basal diet supplemented with 0.5 percent *l*-cystine; group 3 was given the basal diet supplemented with 0.06 percent *p*-dimethylaminoazobenzene, 0.5 percent *l*-cystine, and 0.2 percent choline; and group 4 received the basal diet supplemented with 0.06 percent *p*-dimethylaminoazobenzene and 0.5 percent *dl*-menthionine. The animals were allowed to ingest their respective diets ad libitum. Food intake and body weight were determined semiweekly throughout the course of the experiment. The animals were palpated for hepatic tumors at each weighing after the first 90 days on the diet. Whenever a mass was thus suspected, a record was made and almost without exception a mass would become large enough within 14 days to leave no doubt as to its presence. The animal was killed, and representative sections were taken for microscopic examination. Animals ingesting the basal diet alone or the diet supplemented with cystine were killed periodically beginning at the one hundred and twentieth day. The data, summarized in table 1, are all based on microscopic examination of the tissues obtained at autopsy.

Four of the rats on the basal diet alone developed cirrhosis of the liver, and 21 on the basal diet supplemented with cystine likewise developed this lesion. The diagnosis of cirrhosis of the liver, often suggested by the gross picture (fig. 1, *A*),[1] was made on microscopic examination which revealed distortion of the architecture by strands of connective tissue separating various-sized islands of regenerated parenchyma (figs. 1, *B*, and 2, *A* and *B*). The distribution of the distorted architecture was focal, and the width of the fibrous bands varied considerably (fig. 2). Single or small groups of parenchymal cells were often found in bundles of connective tissue. In some specimens in which the diagnosis of cirrhosis was not made, there were minor

[1] *All photomicrographs in this paper were prepared from sections stained with haematoxylin and eosin, unless otherwise stated.*

distortions of architecture by relatively thin strands of reticulum (fig. 3, *A*).

The livers of both groups 1 and 2 contained a small amount of granular brown pigment which gave a positive reaction for iron with the hydrochloric acid-potassium ferrocyanide test and a large amount of canary-yellow pigment which, although

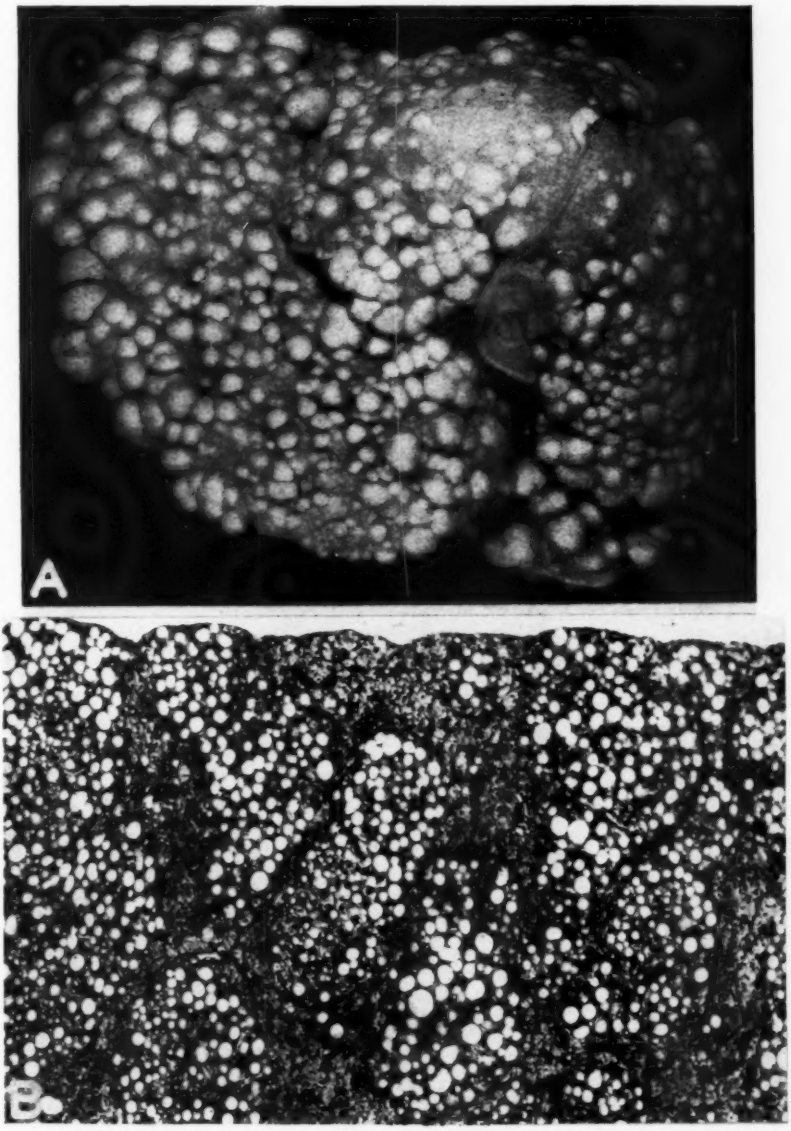


FIGURE 1.—Cirrhosis of the liver of rats on White-Jackson diet, no supplement. *A*, Gross specimen enlarged; *B*, Photomicrograph. × 85.

insoluble in organic solvents, reacted positively for lipoids with sudan IV and with osmic acid. When tested with Verhoeff's stain for acid-fast bacilli, the latter pigment presented an acid-fast reaction. There was a small amount of this pigment within Kupffer cells and in foci of macrophages supported by reticulum in some of the noncirrhotic livers (fig. 3, *B*), but it was consistently present, often in large amounts, in those livers which showed cirrhosis. In these the pigment was

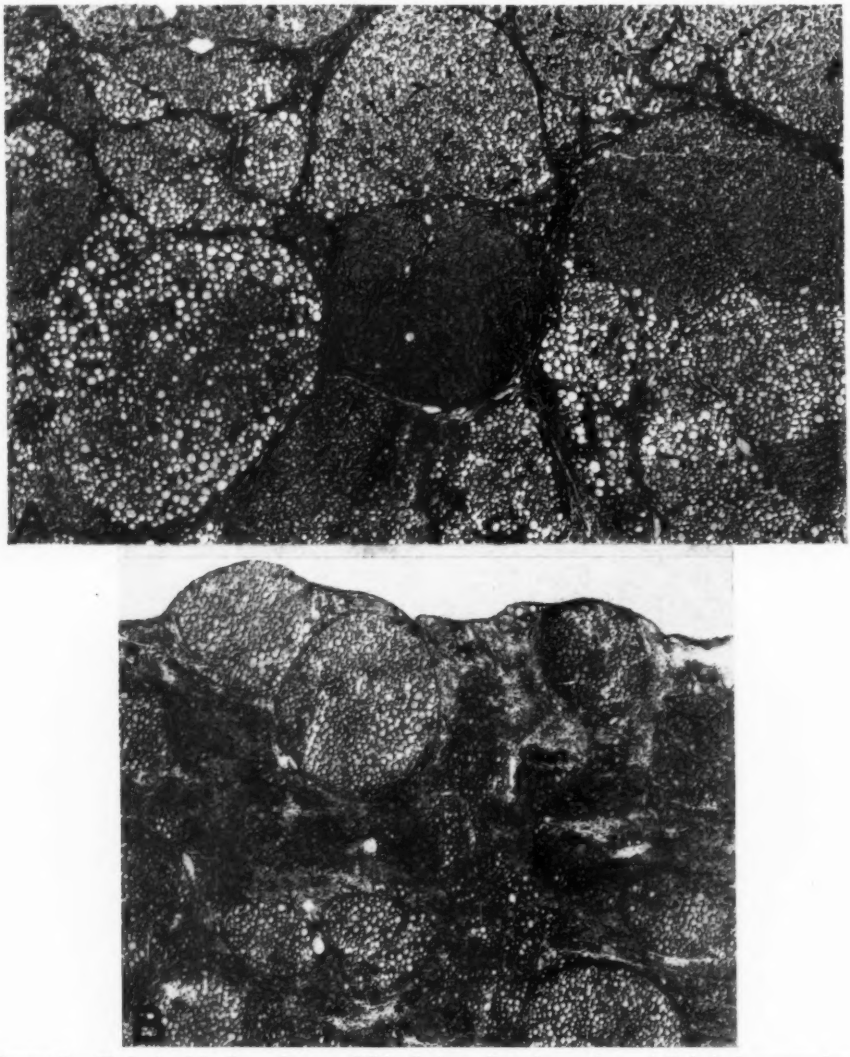

FIGURE 2.—Cirrhosis of the liver of rats on White-Jackson diet, plus cystine. *A*, Relatively narrow bands of connective tissue, and varying amounts of fat. × 45; *B*, Wide bands of connective tissue. × 42.

present in the Kupffer cells but showed particular concentration within phagocytes lying in areas of fibrosis (fig. 4).

The livers of the rats on the *p*-dimethylaminoazobenzene- and on the cystine-*p*-dimethylaminoazobenzene- supplemented diets (groups 5 and 6) commonly showed cirrhosis, with the presence of the two pigments seen in the rats to which *p*-dimethylaminoazobenzene had not been given, that is, those animals of groups 1 and 2. The fibrosis was greater in the animals that received supplementary cystine. In addition to fibrosis and pigmentation, these livers showed areas of nonneoplastic bile duct proliferation and areas of cystic

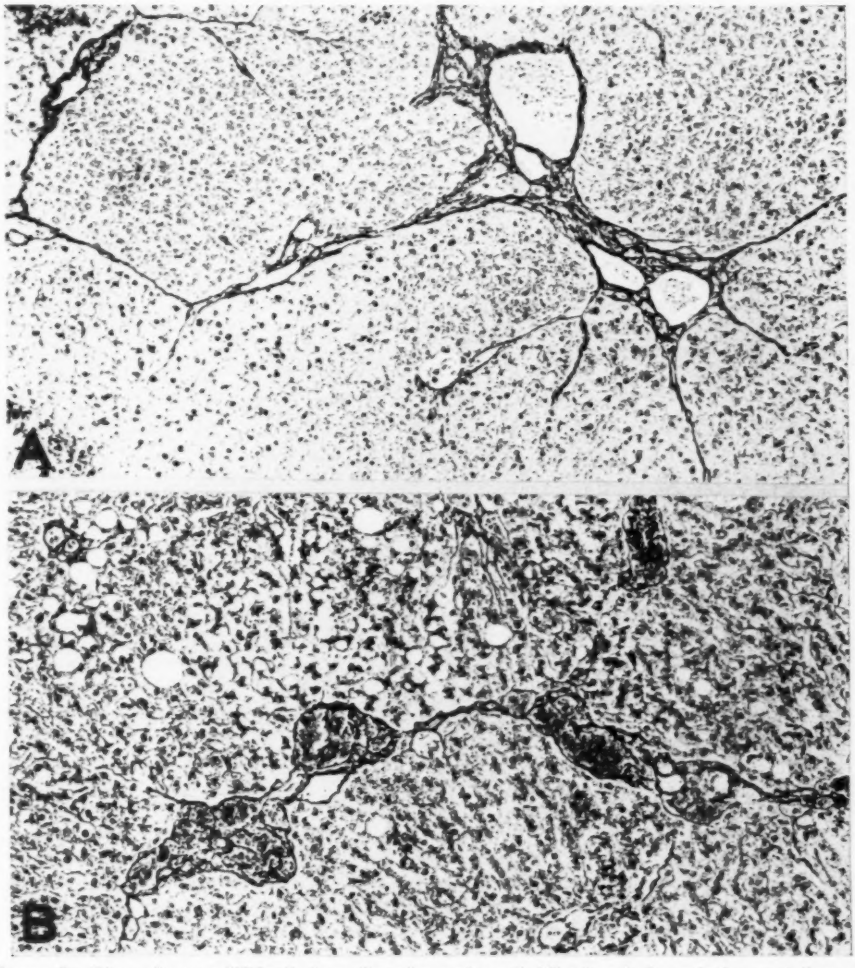


FIGURE 3.—Liver of rats on White-Jackson diet, plus cystine. Laidlow's stain for reticulum. *A*, Increase in portal reticulum with extension into lobule. × 95; *B*, Pigment-laden macrophages supported by reticulum. × 110.

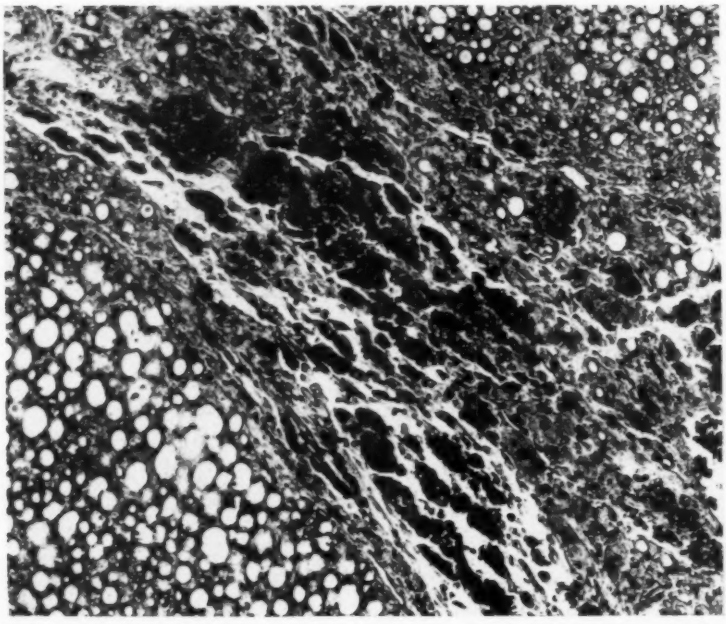

FIGURE 4.—Liver of rats on White-Jackson diet plus cystine. Osmic acid stain on paraffin section of tissue. Osmiophilic pigment with bands of connective tissue. ×145.

dilatation of the bile ducts. Primary malignant epithelial tumors were present in each group in the following distribution: Forty-eight of the eighty rats maintained for 120 to 500 days on the basal diet supplemented with *p*-dimethylaminoazobenzene alone, or 60.0 percent, showed malignant neoplasms of the liver, while in the group receiving the basal diet supplemented with *p*-dimethylaminoazobenzene plus cystine the tumor incidence in 87 rats maintained for 120 to 500 days was 96.5 percent. Reports on the pathology and tumor incidence in the rats included in groups 5 and 6 in this paper have appeared earlier (*12*, *15*).

The livers of the rats on the cystine-choline-*p*-dimethylaminoazobenzene- and on the methionine-*p*-dimethylaminoazobenzene-supplemented diets (groups 3 and 4) were similar in appearance. Grossly, the livers without tumors presented smooth, nongranular capsules, the color of which was grayish brown with specks of yellow. Occasionally, there was a flat-based, gray depression in the capsule or a shrunken area at the margin of a lobe. The consistence of the liver was not appreciably increased, and there was no recognizable disturbance of architecture on examination of the cut surface.

The livers which bore neoplastic tissue were usually highly irregular in shape because of multiple nodules of soft, gray, purple, or yellowish-gray tumor measuring up to 4.0 cm. in diameter. The hepatic tissue intervening between the tumor masses was similar in appearance to the livers in which no tumors were found. In table 1 it can be seen that 27 of 30, or 90 percent, developed hepatic tumors in group 3, and 18 of 20, or 90 percent, in group 4 developed malignant neoplasms.

Microscopically, the livers showed very

little disturbance of lobular architecture. There was, however, an almost consistent increase in reticulum within the hepatic lobule. This was most evident in the portal areas from which there was extension of heavy reticulum fibers into the interior of the lobule (fig. 5). This element not only occupied the usual site for

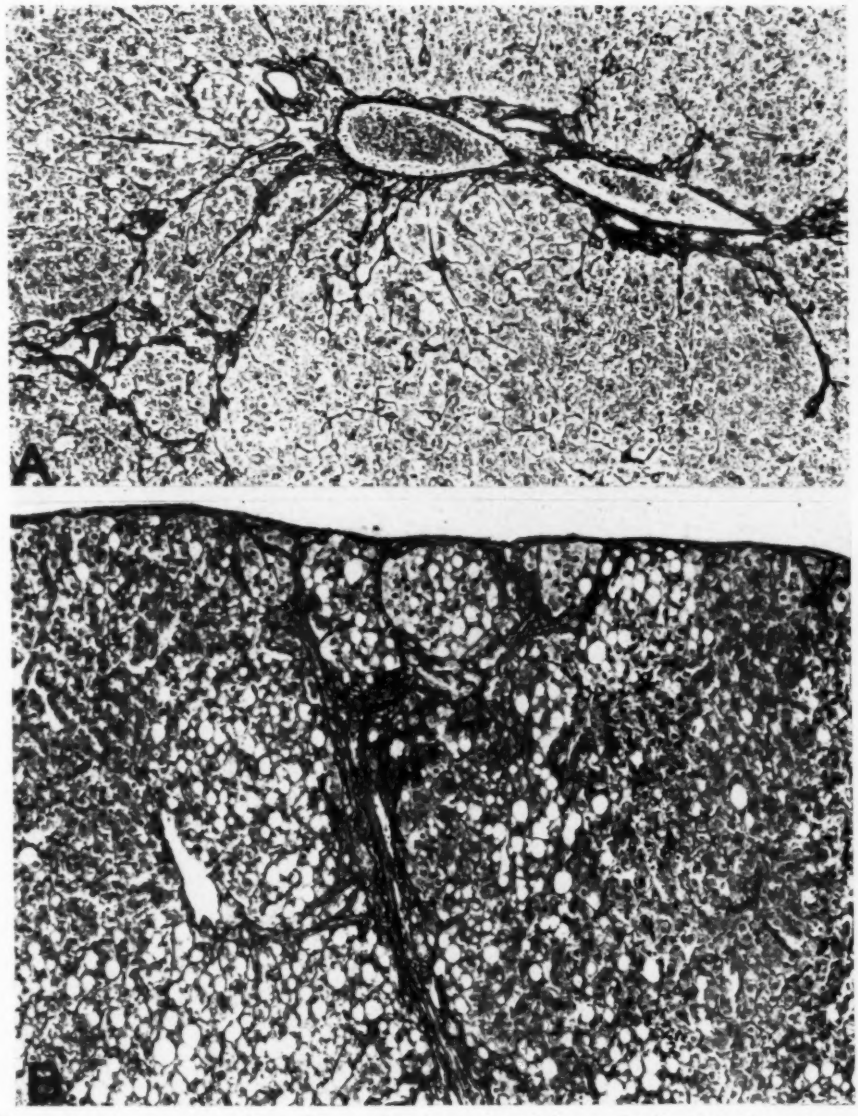


FIGURE 5.—Livers of rats on White-Jackson diet plus cystine, choline, and *p*-dimethylaminoazobenzene. Laidlaw's stain for reticulum. *A*, Increase in intralobular reticulum. × 105; *B*, Reticulum surrounding small islands of parenchyma. × 120.

reticulum in the lobule, that is, between the epithelial cord and the sinusoid, but also encircled individual (fig. 6) or small groups of cells (fig. 5).

A canary-yellow pigment, which gave a positive acid-fast reaction with Verhoeff's stain for tubercle bacilli and the granular iron-positive pigment were found within Kupffer cells and phagocytes lying in portal areas. Areas of cystic bile duct dilatation were seen occasionally (fig. 7, *A*), and bile duct proliferation was not infrequently encountered (fig. 7, *B*). As in earlier experiments with *p*-dimethylaminoazobenzene in the rat (*15*), this lesion varied in extent. In some sites it was restricted to the portal areas, whereas in others it was too extensive to be identified with any portion of any one hepatic lobule. The larger of these lesions often coincided with the depressed or shrunken areas described grossly.

The characteristics which distinguished this lesion from adenocarcinoma were the formation of ductlike spaces lined by flat or cuboidal, well-differentiated, slowly growing cells lacking stratification, fibrosis of older lesions with loss of epithelial elements (fig. 7, *B*), and the presence of the lesion within shrunken areas rather than within a tumor mass. There were no peritoneal implants or metastases reproducing the histologic picture of these proliferated bile ducts.

In contrast with this picture, adenocarcinoma showed acinar structures often lined by columnar cells, and frequent stratification of cells (fig. 10) as well as

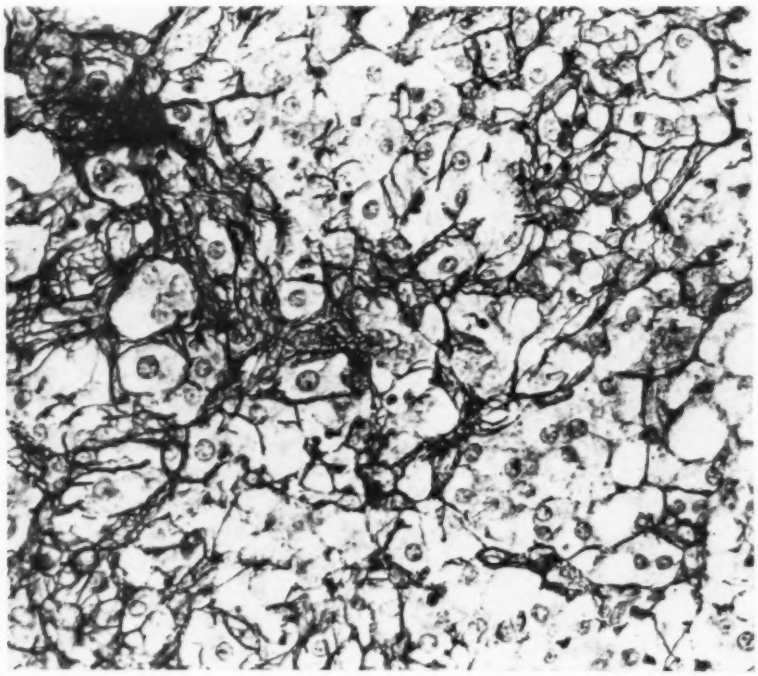

FIGURE 6.—Liver of rat on same diet as that given for rats in legend for figure 5. Laidlaw's stain for reticulum. Increase in intralobular reticulum with encirclement of single cells. × 355.

considerable mitotic activity. This lesion was part of a tumor mass rather than lying within a shrunken area as was the case with bile duct proliferation.

The tumors of the liver were similar to those induced in other experiments and already classified and described (*15*). There were examples of hepatoma types

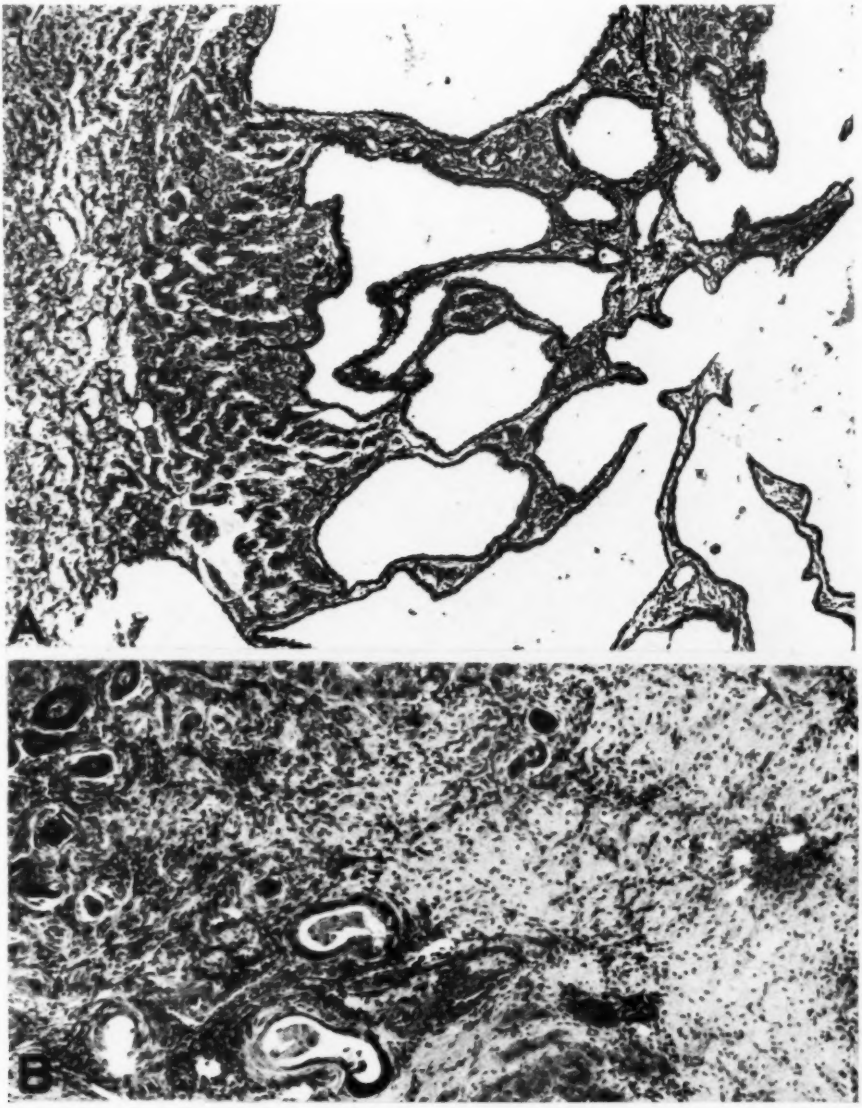


FIGURE 7.—Livers of rats on same diet as that indicated in legend for figure 5. *A*, Cystic dilatation of bile ducts. Laidlaw's stain for reticulum. × 85; *B*, Nonneoplastic bile duct proliferation. Notice area of fibrosis with disappearance of epithelium. × 125.

I and II and of adenocarcinoma. The tumors classified as hepatoma showed a fundamental liverlike architecture in which epithelial cords of varying width alternated with endothelial-lined sinuses. In the tumor areas subdivided into hepatoma type I, the tumor cells were highly differentiated, parenchymalike in appearance,

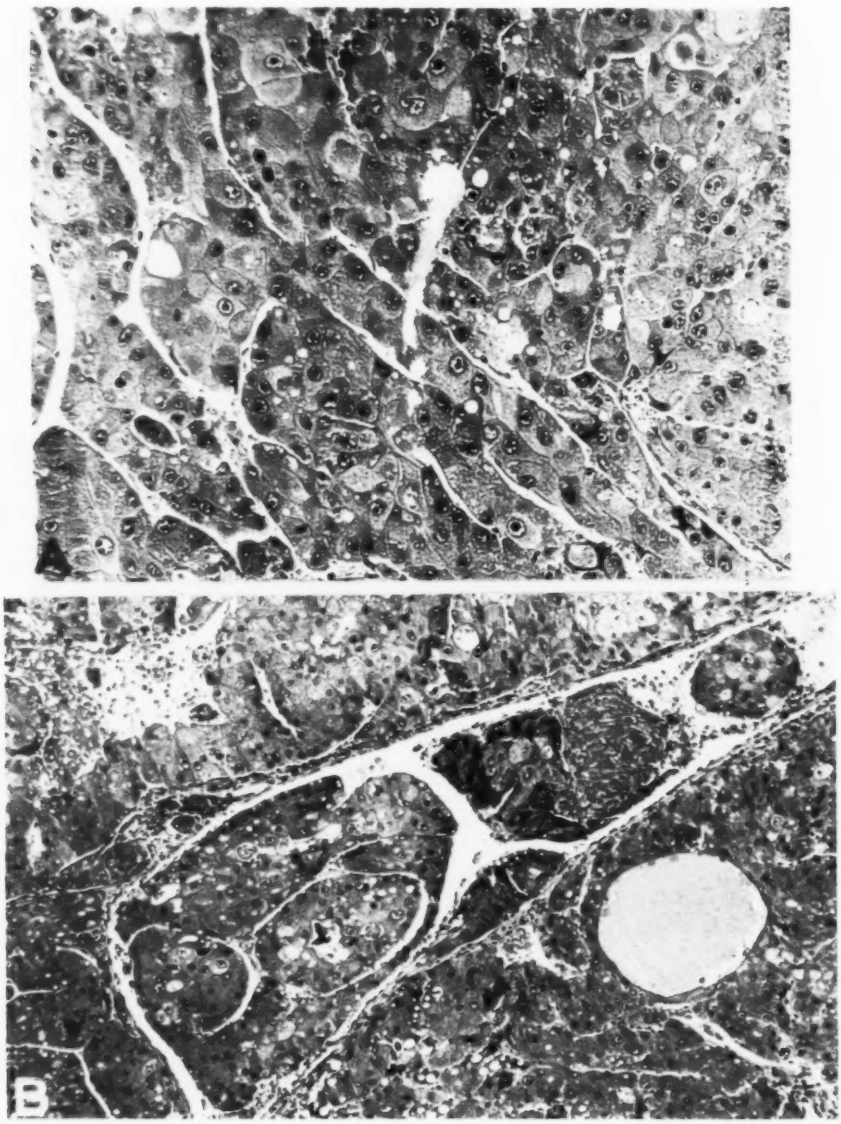


FIGURE 8.—Livers of rats on White-Jackson diet plus cystine, choline, and *p*-dimethylaminoazobenzene. *A*, Hepatoma type I. × 180; *B*, Invasion of blood vessel by hepatoma type I. × 105.

with acidophilic cytoplasm, large vesicular nuclei with prominent nucleoli, and prominent cell membranes (fig. 8, *A* and *B*). Even this highly differentiated form showed invasion of blood vessels (fig. 8, *B*). Hepatoma type II was characterized by cells with basophilic cytoplasm and indistinct cytoplasmic membranes forming varying-sized cords alternating with blood sinuses (fig. 9). The adenocarcinomas showed

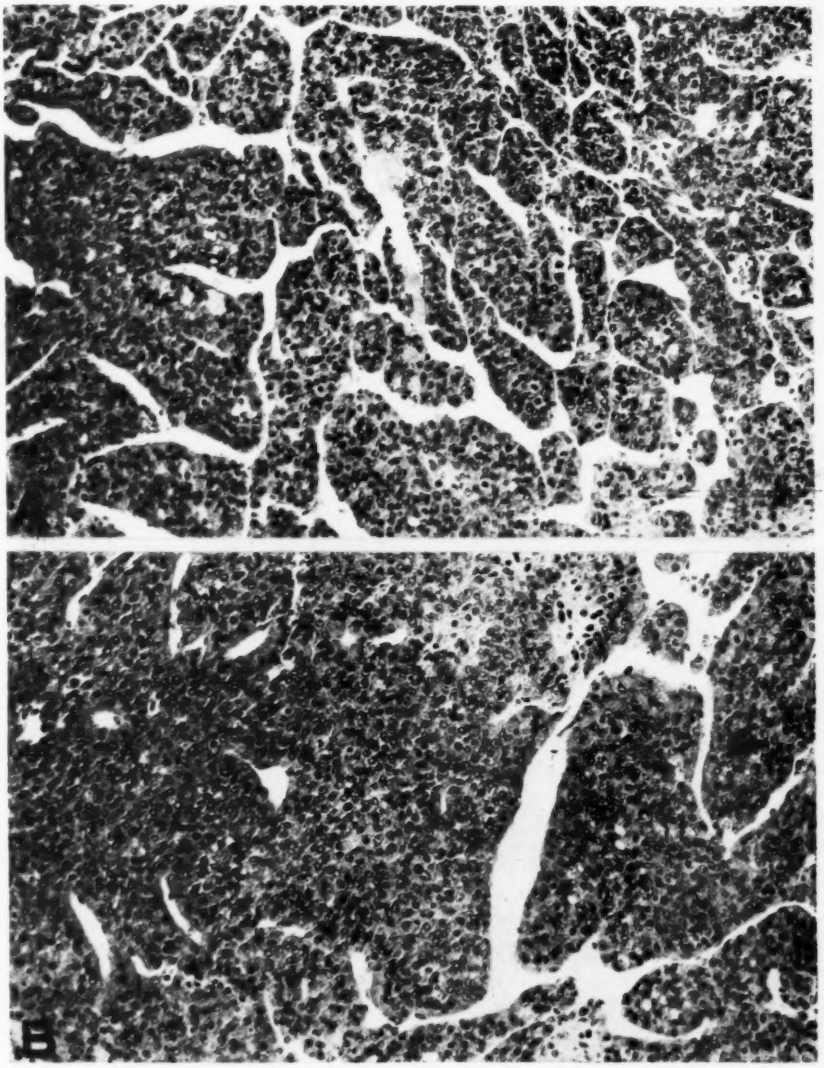


FIGURE 9.—Livers of rats on White-Jackson diet plus cystine, choline, and *p*-dimethylaminoazobenzene. *A*, Hepatoma type II with relatively narrow cords. × 180; *B*, Hepatoma type II with relatively wide cords. × 180.

no characteristic organlike structure (fig. 10). The tumor cells formed acinar structures and as already indicated were often columnar, stratified, and showed numerous mitotic figures. Adenocarcinoma was not encountered alone, being constantly associated with hepatoma, particularly type II. While sometimes the

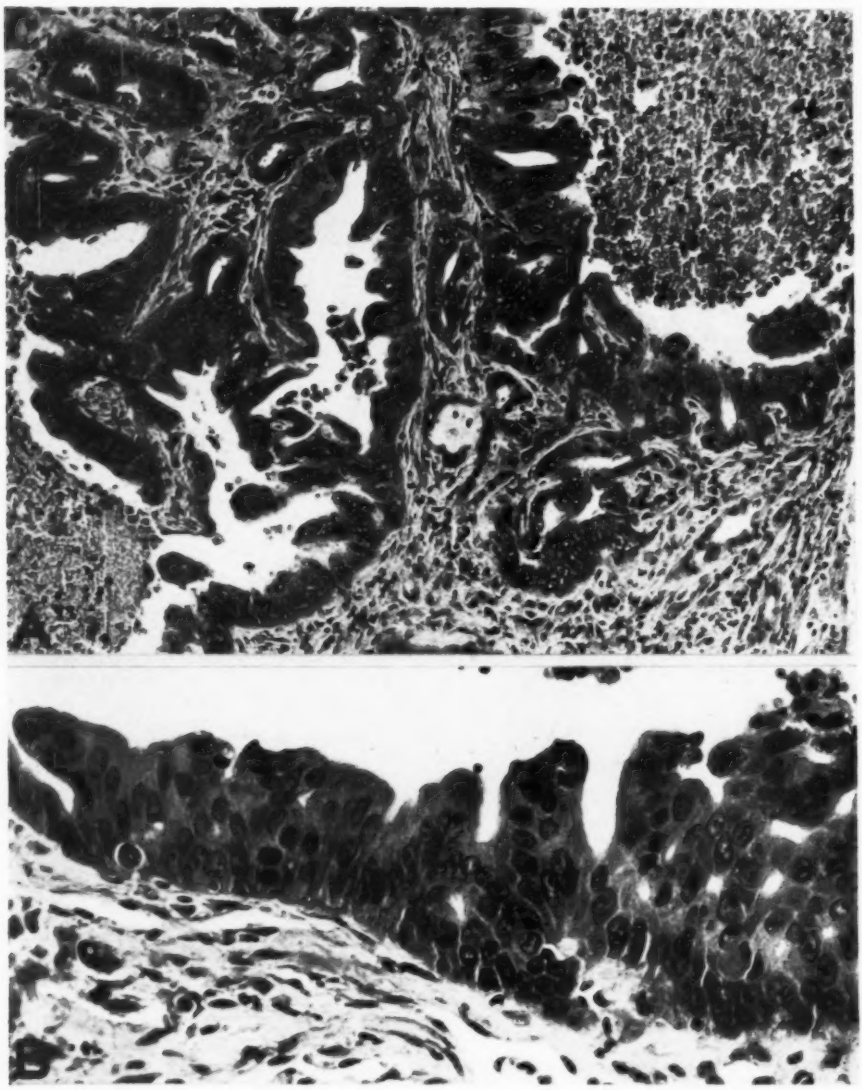

FIGURE 10.—Livers of rats on White-Jackson diet plus cystine, choline, and *p*-dimethylaminoazobenzene. *A*, Adenocarcinoma. Note lack of characteristic liverlike structure shown by the hepatomas (figs. 8 and 9). × 180; *B*, Adenocarcinoma. Note stratification of cells forming acinus. Contrast with bile duct proliferations (fig. 7, *B*). × 430.

association of adenocarcinoma and hepatoma was in the form of a collision tumor, there were instances in which transitions could be distinguished between hepatoma type II and adenocarcinoma (fig. 11).

## DISCUSSION

Under the experimental conditions reported, it appears that the addition of cystine and choline has no appreciable effect on the incidence of hepatic tumors in rats receiving *p*-dimethylaminoazobenzene as compared with rats receiving the basal diet plus cystine and *p*-dimethylaminoazobenzene but no choline. Methionine, which is considered lipotropic and which should behave in a manner analogous to choline, likewise had no definite effect on the incidence of induced hepatic tumors. These findings do not support the conclusions reported by György, Poling, and Goldblatt (*11*) that the addition of cystine and choline will markedly retard the induction of malignant hepatic tumors produced by a diet containing *p*-dimethylaminoazobenzene.

The diets used by György, Poling, and Goldblatt to study the effect of choline and cystine on the production of hepatic tumors by *p*-dimethylaminoazobenzene were identical with ours except for the fact that we used dried brewers' yeast as a source of the B vitamins and they used crystalline supplements. Furthermore, their experiment lasted up to 175 days. It would be interesting to know whether tumors would have developed had the experiment continued beyond 175 days. György and Goldblatt (*7*) also reported that rats kept on the diet of White and Jackson (*13*) and supplemented with 0.5 gm. yeast three times a week resulted in no hepatic injury. The period of experimentation was not stated. We have found contrariwise that rats on the same White-Jackson diet supplemented with 0.4 gm. of yeast daily would show evidence of hepatic injury as early as 8 months but usually about the fifteenth month. We have observed that the addition of 0.5 percent *l*-cystine to the above diet increases the incidence of cirrhosis of the liver and that the changes appear as early as 5 months and are common in rats that reach an age of 14 months. The difference in appearance of the livers in the groups receiving choline plus cystine or methionine from the livers of rats which had not been given supplementary choline or methionine is in line with work in which cirrhosis was reported as being prevented when rats receiving a basal diet low in protein and high in fat were given supplementary choline or methionine (*8*).

It should be emphasized that the livers of our rats receiving supplementary choline or methionine were not entirely normal. Grossly they failed to present the picture of cirrhosis, and microscopically when examined with ordinary stains they frequently gave little evidence of any disturbance in architecture. With stains for demonstrating connective tissue an appreciable increase in intralobular connective tissue or reticulum was often observed, which evidenced injury.

Occasionally the reticulum set off areas of parenchyma into small, irregular lobules, but more often it was confined to the space between the sinusoid and the epithelial cord or encircled individual parenchymal cells of the lobule. While these alterations are admittedly minor in degree anatomically, we are not prepared to say that, to the specific cells affected, they are of equally minor importance functionally.

Nonneoplastic bile duct proliferation and bile duct dilatation were lesions seen exclusively in the rats belonging to the groups receiving *p*-dimethylaminoazobenzene. They were not observed in the animals receiving the basal diet alone or

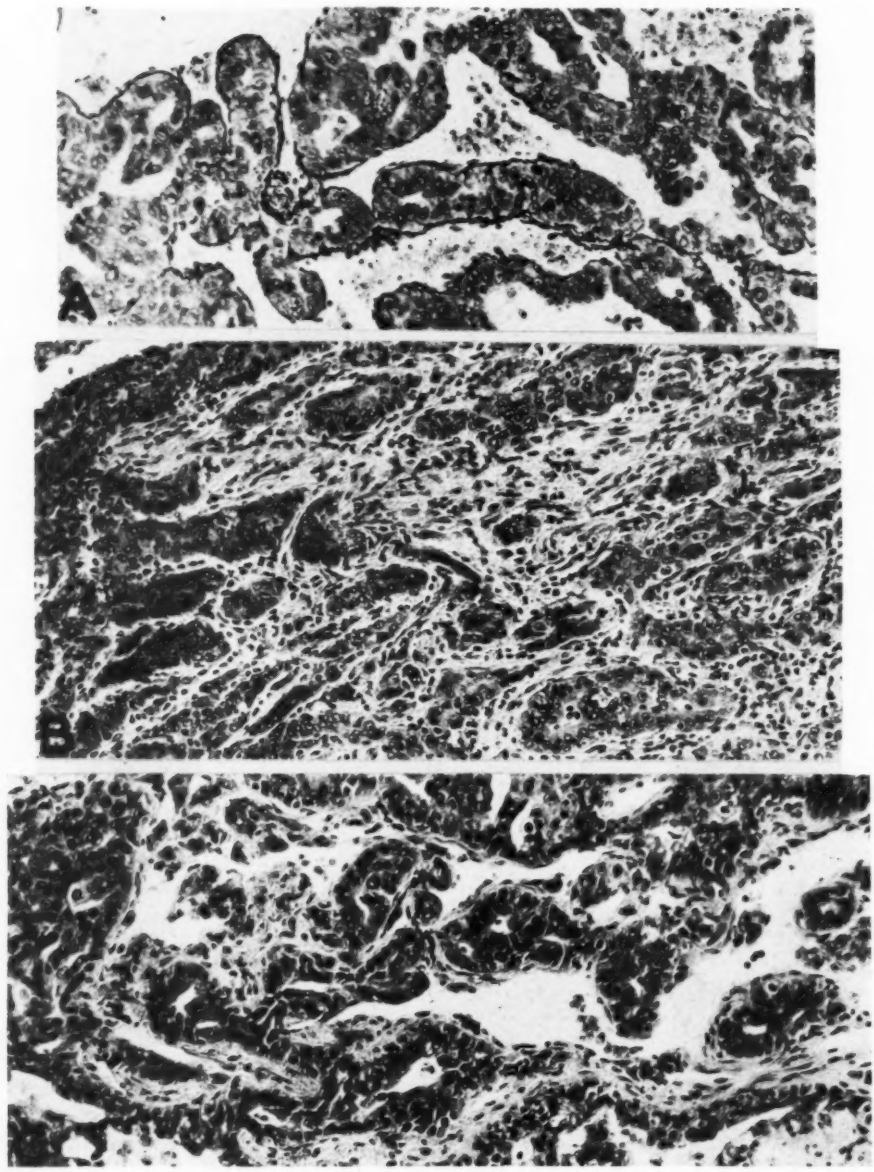


FIGURE 11.—Livers of rats on White-Jackson diet plus cystine, choline, and *p*-dimethylaminoazobenzene. Variations in connective tissue of hepatoma type II. × 180. *A*, Usual picture of fine fiber of reticulum between epithelial cord and sinusoid. Notice acinar structures in cords. Laidlaw's stain for reticulum; *B*, Abundant connective tissue separating cords of tumor; *C*, Varying amounts of connective tissue in relation to acinar hepatoma cords. Towards the left, the association of acinar hepatoma cords with abundant connective tissue produces an adenocarcinomatous structure.

supplemented with cystine. It is of importance to recognize the nonneoplastic nature of the proliferated bile ducts since to consider them as neoplasms may be responsible for faulty interpretation of results in experiments concerned with induction of tumors where animals showed only this lesion. In an earlier paper we reviewed evidence for and against considering this lesion to be nonneoplastic rather than an adenocarcinoma (*15*). We arrived at the conclusion, which we still hold, that the bile duct proliferation under discussion is not a neoplasm. The features which distinguish this lesion from a neoplasm are its relatively slow growth, high degree of differentiation of the cells, lack of stratification of cells, its presence in shrunken areas in the liver rather than in tumor masses, the progressive atrophy and fibrosis, lack of metastasis, and failure to obtain successful transplants (*15*).

In the earlier paper (*15*) we reported on the presence of pigments in the livers of rats on the basal diet of White and Jackson supplemented with *p*-dimethylaminoazobenzene either with or without the addition of cystine. The results of experiments recorded here indicate that at least some of the pigmentation must be attributed to the influence of the basal diet rather than to *p*-dimethylaminoazobenzene alone. In support of this idea is the pigmentation of the livers in rats of groups 1 and 2, neither of which received *p*-dimethylaminoazobenzene. While it is our feeling that the iron-containing pigment was more common in the groups of rats receiving *p*-dimethylaminoazobenzene, the non-iron-containing pigment which gave the staining reactions of lipoids was fully as abundant in rats receiving only the basal diet with or without a supplement of cystine. In 1941 we discussed the nature of this latter pigment and suggested that it might be a conjugated lipoid since it gave the staining reactions for lipoids with osmic acid, sudan IV, and Verhoeff's stain for acid-fast bacilli on the one hand but failed to dissolve in organic solvents on the other (*15*, *20*). In the same year similar pigment was reported by Blumberg and Grady (*16*) in the livers of rats receiving high fat-low protein diets. More recently Lillie and coworkers (*17*) described similar reactions for a pigment which occurred in the livers of rats on a low protein diet, and in this laboratory a similar pigment was observed in mice on adequate diets receiving either carbon tetrachloride (*18*) or *o*-aminoazotoluene (*19*). From the variety of means by which this substance may be obtained one is led to the suspicion that it is derived in some way from disintegrated hepatic cells.

## SUMMARY

Four of thirty Osborne-Mendel rats maintained on a basal low protein-high fat diet (White-Jackson) developed cirrhosis of the liver, and 21 of 30 rats on the basal diet supplemented with 0.5 percent *l*-cystine showed this lesion. No hepatic tumors developed in either group.

Of 87 animals on the basal diet supplemented with 0.5 *l*-cystine and 0.06 percent *p*-dimethylaminoazobenzene, 96.5 percent developed primary malignant hepatic tumors, and of 80 rats on the basal diet with a supplement of 0.06 percent *p*-dimethylaminoazobenzene but with no added cystine, 60.0 percent showed similar tumors. The rats of these two groups showed a high incidence of cirrhosis of the liver which was more extensive in the group receiving supplementary cystine.

In a group of 30 rats receiving the basal diet supplemented with 0.06 percent *p*-dimethylaminoazobenzene, 0.5 percent *l*-cystine, and 0.2 percent choline, the incidence of primary malignant tumors of the liver was 90.0 percent, and a similar incidence

was obtained in a group of 20 animals maintained on the basal diet supplemented with 0.06 percent *p*-dimethylaminoazobenzene and 0.5 percent methionine. The addition of choline plus cystine or methionine to the basal diet containing *p*-dimethylaminoazobenzene had no apparent effect on the incidence of hepatic tumor formation. The animals of these two groups did not show any extensive cirrhosis of the liver but did show an almost constant increase in intralobular connective tissue or reticulum which often encircled single parenchymal cells.

Two pigments were present in the livers of all the groups of rats. The first was brown, granular, and iron-containing. It appeared to be more abundant in the animals receiving *p*-dimethylaminoazobenzene. The second pigment was globular, canary-yellow in color, and although insoluble in organic solvents, had a positive reaction for lipoids with sudan IV and osmic acid. In addition, it gave a brilliant acid-fast reaction with Verhoeff's stain for acid-fast bacilli. Because these pigments were observed in all groups of animals including those that did not receive *p*-dimethylaminoazobenzene, the basal diet must be held responsible, at least in part, for their presence.

Nonneoplastic bile duct proliferation and cystic bile duct dilatation were observed only in those rats receiving *p*-dimethylaminoazobenzene. This compound is, therefore, considered as the agent responsible for the induction of this lesion. It is important to distinguish nonneoplastic bile duct proliferation from adenocarcinoma. The differential diagnosis is given.

The pathology of the individual tumors and hepatic lesions are described and illustrated.

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# Sulfhydryl Groups in Normal and Tumorous Hepatic Tissue Extracts Before and After Addition of Salts

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## INTRODUCTION

Native, unaltered proteins, with very few exceptions, do not contain free titratable sulfhydryl groups. When, however, these proteins are denatured by various agents, such as heat, ultraviolet irradiation, or salts, they give evidence, also with few exceptions, of the presence of free titratable sulfhydryl groups. The reason for the appearance of these and, presumably, a number of other kinds of groups is that the protein molecule unfolds as a result of the denaturation. The sulfhydryl groups, which are probably bound in a nonreactive form within the relatively rigid configuration of the native protein, are released when the configuration of the latter is destroyed as a result of the denaturation, and the groups then become available to added titrating agents. Since the sulfhydryl groups have reducing properties, the usual type of titrating agent is an oxidant, although substituting agents, such as iodoacetamide, have also been employed. The titrating agent used is standardized against known solutions of cysteine, and the values found with proteins are then given in terms of cysteine.

This, in brief, is the basis of a number of investigations which have been conducted during the past decade on the mercaptan groups of proteins and the effect of denaturants. Among the titrating agents used for these groups have been the following: chlorindophenol, phosphotungstic acid, ferricyanide, mercuribenzoate, iodosobenzoate, and porphyrindin. The denaturing agents in most common use at the present time are the guanidine salts introduced by Greenstein (*1–4*) and the detergents introduced by Anson (*5*). A review and comparison of the data obtained on well-defined proteins by the use of the denaturing salts and the titrating agents has recently been made by Greenstein and Jenrette (*6*). On the whole, the results by different investigators using different techniques have been concordant. The work of Edsall and Mehl (*7*) and of Neurath and his coworkers (*8–10*) on the effect of various denaturants on the physical properties of the proteins have shown that the magnitude of the change in these properties as a result of denaturation runs parallel to the number of sulfhydryl groups that appear.

An application of this type of investigation to proteins isolated from tumor tissues was made by Greenstein, Jenrette, and White (*11*), who estimated the free sulfhydryl-group content of the native nucleoprotein fraction from rat hepatoma 31 transplants and the sulfhydryl groups liberated in this protein fraction after denaturation with guanidine hydrochloride. Comparison with the results obtained in the same manner with the nucleoprotein fraction of normal rat liver revealed no difference between the two tissues.

The nucleoprotein fraction of the hepatoma and of the liver represented only a small fraction of the total proteins of these tissues. It was thought desirable to extend these studies to the measurement of

the sulfhydryl-group content of the whole water-extractable proteins of these and other tissues before and after the addition of salts to the extract. A certain complication was encountered in the studies of the extracts because of the probable presence of glutathione in the latter. The sulfhydryl groups measured in the aqueous extracts before the addition of salts may be the sum of those belonging to the glutathione and to the free groups of the native proteins. On the other hand, if the sulfhydryl-group content of the extracts increases after the addition of the salts, it can be stated that this increase is due to the appearance of mercaptan groups in the proteins as a result of denaturation.

## EXPERIMENTAL PROCEDURE

### Tissues

The tissues employed included the following: rat hepatoma 31 (*12*) in the ninth, tenth, twenty-ninth, thirtieth, and thirty-first transplants, hepatoma 587 in strain A mice in the third transplant (*13*), hepatoma 98/15 in strain C3H mice in the fifth transplant (*13*), and hepatoma A in strain C3H mice (*14*). Hepatomas 587 and 98/15 were donated for these studies by Dr. Jesse E. Edwards, and hepatoma A by Dr. J. W. Thompson, of this Institute. The two mouse tumors first mentioned had originally arisen spontaneously in mice of the respective strains, and the last-mentioned tumor was originally induced by repeated injections of *o*-aminoazotoluene. We have, therefore, in the case of the mouse hepatomas two tumors of spontaneous origin in two different strains of mice, and two tumors in the same strain (C3H), one of which arose spontaneously while the other was originally induced by a carcinogenic chemical. Other tissues included normal and regenerating rat liver, normal mouse liver, the livers of hepatoma-bearing rats and mice, normal adult rabbit liver, and fetal rabbit liver.

The tissues were removed immediately after the death of the animal (generally by decapitation) and ground in a mortar with sand. The pulp was taken up in three times the weight of distilled water and allowed to stand for a given length of time in the ice box. At the end of this time the extract was centrifuged for 5 minutes at 1500 r. p. m. and the supernatant decanted. The total nitrogen was determined on an aliquot of the supernatant, which was distributed in 1-cc. portions between two sets of small test tubes. One set was set aside, and to the other various salts were added. Within a period of 15 minutes from the time that the original extract had been removed from the ice box, the solutions in the two sets of test tubes were titrated with porphyrindin and nitroprusside in the manner already fully described (*1–4, 6, 15, 16*). It was found that consistent results could be obtained only on freshly prepared tissues, for tissues kept frozen for even a few days and then extracted yielded variable results. The porphyrindin was standardized against cysteine. The results on the extracts are given, therefore, in terms of cysteine. The data on extracts of normal rat liver and of rat hepatoma 31 transplants are given in table 1.

Controls run on the salts studied revealed no reducing properties. The various salts were employed at different concentrations. The column "Extraction time" refers to the time in which the extract of ground tissue and water was allowed to remain in the ice box. The column "Free cysteine" refers to the sulfhydryl groups as cysteine per milligram total nitrogen estimated in the centrifuged extracts before the addition of the salts. The column "Total cysteine" re-

TABLE 1.—*Effect of added agents on extracts of rat hepatoma 31 transplants and of normal rat liver*

[1.0 cc. extract titrated with porphyrindin (32.5 mg. per 100 cc. $H_2O$) before and after addition of agents]

| Salt | Concentration of salt | Extraction time | Age of tumor [1] | "Free cysteine" | | "Total cysteine" | |
|---|---|---|---|---|---|---|---|
| | | | | Liver | Tumor | Liver | Tumor |
| | *mM per cc. extract* | *Hours* | *Days* | *Mg. per mg. total N* | *Mg. per mg. total N* | *Mg. per mg. total N* | *Mg. per mg. total N* |
| Guanidine hydrochloride | 3 | 1 | 14 | 0.042 | 0.034 | 0.100 | 0.056 |
| | 9 | 1 | 14 | .042 | .034 | .100 | .056 |
| | 16 | 1 | 14 | .042 | .034 | .100 | .056 |
| | 3 | 1 | 35 | .043 | .029 | .103 | .055 |
| | 9 | 1 | 35 | .043 | .029 | .103 | .055 |
| | 16 | 1 | 35 | .043 | .029 | .103 | .055 |
| | 3 | 1 | 49 | .045 | .035 | .104 | .050 |
| | 9 | 1 | 49 | .045 | .035 | .104 | .050 |
| | 16 | 1 | 49 | .045 | .035 | .104 | .050 |
| | 9 | .25 | 35 | .021 | .018 | .074 | .025 |
| | 9 | 1 | 35 | .043 | .030 | .103 | .045 |
| | 9 | 3 | 35 | .034 | .022 | .083 | .040 |
| | 9 | 6 | 35 | .034 | .020 | .074 | .038 |
| | 9 | 22 | 35 | .011 | 0 | .064 | .021 |
| Guanidine thiocyanate | 6 | 1 | 49 | .047 | .032 | .104 | .050 |
| Guanidine acetate | 6 | 1 | 49 | .047 | .032 | .083 | .041 |
| Guanidine carbonate | 6 | 1 | 49 | .047 | .032 | .062 | .038 |
| Guanidine sulfate | 6 | 1 | 49 | .047 | .032 | .075 | .040 |
| Lithium carbonate | 1 | 1 | 49 | .047 | .032 | .063 | .028 |
| Lithium chloride | 19 | 1 | 49 | .047 | .032 | .100 | .060 |
| Lithium sulfate | 3 | 1 | 49 | .047 | .032 | .104 | .055 |
| Sodium fluoride | 1 | 1 | 49 | .047 | .032 | .092 | .038 |
| Sodium chloride | 6 | 1 | 49 | .047 | .032 | .100 | .056 |
| Sodium bromide | 9 | 1 | 49 | .047 | .032 | .103 | .060 |
| Sodium iodide | 13 | 1 | 49 | .047 | .032 | .100 | .064 |
| Sodium thiocyanate | 18 | 1 | 49 | .047 | .032 | .100 | .050 |
| Sodium carbonate | 1 | 1 | 49 | .047 | .032 | .081 | .035 |
| Sodium acetate | 14 | 1 | 49 | .047 | .032 | .080 | .034 |
| Sodium sulfate | 1 | 1 | 49 | .047 | .032 | .063 | .020 |
| Potassium chloride | 5 | 1 | 49 | .047 | .032 | .108 | .060 |
| Potassium bromide | 6 | 1 | 49 | .047 | .032 | .108 | .060 |
| Potassium iodide | 10 | 1 | 49 | .047 | .032 | .108 | .060 |
| Potassium thiocyanate | 18 | 1 | 49 | .047 | .032 | .108 | .060 |
| Potassium carbonate | 11 | 1 | 49 | .047 | .032 | .073 | .030 |
| Potassium acetate | 30 | 1 | 49 | .047 | .032 | .084 | .030 |
| Potassium sulfate | .6 | 1 | 49 | .047 | .032 | .074 | .032 |
| Calcium chloride | 7 | 1 | 49 | .047 | .032 | .100 | .050 |
| Calcium bromide | 10 | 1 | 49 | .047 | .032 | .081 | .040 |
| Calcium iodide | 7 | 1 | 49 | .047 | .032 | .090 | .043 |
| Magnesium chloride | 10 | 1 | 49 | .047 | .032 | .088 | .042 |
| Magnesium sulfate | 4 | 1 | 49 | .047 | .032 | .080 | .038 |
| Ammonium chloride | 8 | 1 | 49 | .047 | .032 | .042 | .032 |
| Ammonium bromide | 10 | 1 | 49 | .047 | .032 | .042 | .032 |
| Ammonium iodide | 13 | 1 | 49 | .047 | .032 | .042 | .032 |
| Ammonium thiocyanate | 18 | 1 | 49 | .047 | .032 | .043 | .033 |
| Ammonium acetate | 20 | 1 | 49 | .047 | .032 | .043 | .033 |
| Ammonium carbonate | .9 | 1 | 49 | .047 | .032 | .045 | .030 |
| Ammonium sulfate | 6 | 1 | 49 | .047 | .032 | .044 | .030 |
| Potassium dihydrogen phosphate + dipotassium hydrogen phosphate (equimolar) | 2 | 1 | 49 | .047 | .032 | .042 | .032 |
| Sucrose | 3 | 1 | 49 | .047 | .032 | .044 | .034 |
| Urea | 10 | 1 | 49 | .047 | .032 | .080 | .045 |

[1] Age of tumor refers to time elapsed since implantation of the tumor.

fers to the sulfhydryl groups as cysteine per milligram total nitrogen estimated in these extracts after the addition of salts. Obviously, the difference between the free and total values for any one tissue is a measure of the mercaptan groups which appear in the proteins of the extract as a result of the denaturing action of the salt. With the exception of urea and the guanidine salts, all the salts were used at concentrations approaching saturation. Precipitation of the proteins of the extracts occurred when the calcium and magnesium salts were added. The effect of the added alkali carbonates is not due to the pH produced by solutions of these compounds but to their salt nature, since addition of sodium or potassium hydroxide to the same pH as that found in solutions of the salts does not cause any increase in

the sulfhydryl group titer. The reproducibility of the values for all the determinations was within 10 percent.

The effect of added guanidine hydrochloride on a number of normal and tumorous hepatic tissues is given in table 2.

TABLE 2.—*Effect of added guanidine hydrochloride on extracts of hepatic tissues*

[1.0 cc. extract titrated with porphyrindin (32.5 mg. per 100 cc. $H_2O$) before and after addition of guanidine hydrochloride to concentration of 9 mM per cubic centimeter of extract. Time of extraction, 1 hour]

| Tissue | "Free cysteine" | "Total cysteine" |
|---|---|---|
| | *Mg. per mg. total N* | *Mg. per mg. total N* |
| Normal rat liver | 0.045 | 0.108 |
| Livers of rats carrying hepatoma 31 transplants | .042 | .103 |
| Regenerating rat liver: | | |
| 2 days after removal of lobes | .039 | .101 |
| 4 days after removal of lobes | .041 | .099 |
| Hepatoma 31 transplants in rats | .035 | .055 |
| Necrotic portion of hepatoma 31 | 0 | .012 |
| Normal strain A mice livers | .046 | .128 |
| Normal strain C3H mice livers | .042 | .132 |
| Livers of strain A mice carrying hepatoma 587 transplants | .041 | .130 |
| Livers of strain C3H mice carrying hepatoma 98/15 transplants | .041 | .133 |
| Livers of strain C3H mice carrying hepatoma A transplants | .046 | .127 |
| Hepatoma 587 transplants in strain A mice | .042 | .120 |
| Hepatoma 98/15 transplants in strain C3H mice | .040 | .124 |
| Hepatoma A transplants in strain C3H mice | .040 | .132 |
| Normal adult rabbit liver | .095 | .300 |
| Fetal rabbit liver (19 days) | 0 | .016 |

The ease and accuracy of the titration of the untreated liver extracts are considerably enhanced if the livers are perfused with distilled water before removing them from the animals. When this is not done, the reddish color in the extracts makes the nitroprusside end point difficult to visualize, and hence a certain amount of reliance must be placed on the porphyrindin end point itself. On the other hand, the denaturing salts cause a shift in the absorption of the hemin compounds in such a manner that the extract generally takes on a brownish color. The color developed by mercaptan plus nitroprusside is easily visible in the extract solutions containing denaturing salts.

## DISCUSSION

The data in table 1 show that the free sulfhydryl-group content of the hepatoma 31 transplants is consistently lower by about 24 percent than that of normal rat liver. When the extracts of these tissues are treated with a wide variety of salts, additional sulfhydryl groups appear, the extent of the increase under the same conditions being much greater in the case of the liver (133 percent) than of the hepatoma (64 percent). The age of the tumor, within the limits studied, has no apparent influence on this effect. Within the range of 3–16 mM of added guanidine hydrochloride per cubic centimeter of extract, the effect is independent of the concentration of this salt. When various extraction times are studied, it is observed that if the extract of ground tissue and water stands too long (22 hours), the mercaptan titer is greatly decreased. Oxidation probably accounts for this. Too short a period of standing results in incomplete extraction. The maximum proportion of mercaptan groups appears after about an hour of extraction.

Nearly all of the salts used release mercaptan groups in the proteins in the extracts of liver and of hepatoma. Significant exceptions are all the ammonium salts studied and phosphate. Sucrose also exerts no denaturing effect. Of the effective salts, i. e., those which when added to extracts cause an increase in the number of sulfhydryl groups, the halides appear to be most active. The somewhat lower values found for the salts of the divalent metals may have been due to the coagulation of the proteins in the extract, with the consequent masking of some of the groups. With the exception of the guanidine salts, no attempt was made to compare the various salts at a single concentration. The salts were used close to saturation in order to observe effects

certain to be maximal under the conditions employed. Future work must be concerned with the use of minimal quantities of salts. However, it is interesting to observe the great differences between the denaturing effects of salts such as sodium fluoride at 1 mM or potassium chloride at 5 mM per cubic centimeter on the one hand, and ammonium salts as high as 18 mM per cubic centimeter on the other.

Greenstein and Edsall (*15*) and Edsall and Mehl (*7*) have pointed out in a study of the effect of denaturing salts on myosin that ammonium salts do not liberate additional sulfhydryl groups in this protein and that the inorganic halides exert a more profound effect upon the physical properties of the protein than do ammonium salts. The failure to find any denaturing effect on the proteins of the hepatic tissue extracts by phosphate (table 1) is paralleled by the similar investigation on myosin (*7*, *15*). Even at the high concentration used, it would have been surprising to find a denaturing effect exerted by this ion in view of its widespread use as a buffer in enzyme reactions.

The values for "free cysteine" in the liver and hepatoma are, respectively, about 0.045 and 0.034 mg. cysteine per milligram total nitrogen in the extract (table 1). In terms of milligrams of glutathione per 100 gm. of wet weight of the respective tissues, these values would be 159.1 and 120.6. The value of 159.1 for normal rat liver is within the range of 135–204 found in deproteinized filtrates of this tissue by Thompson and Voegtlin, who used an iodometric titration and reported their results in terms of glutathione (*17*).[1] Other values reported for glutathione in normal rat liver are 171 (*18*) and 220 (*19*). These values for normal rat liver are all of the same order of magnitude. Kinosita's value for the glutathione content of normal rat liver was about 270 mg. per hundred grams of tissue (*20*). Kinosita reported that the glutathione content of the primary hepatoma induced in rats by butter-yellow feeding was about 11 percent lower than that of normal rat liver. The value of the "free cysteine" of the rat hepatoma transplants in the present study is about 24 percent lower than that of normal rat liver. It is possible that what has been termed "free cysteine" in tables 1 and 2 of this paper may be glutathione. The inference receives further support by converting the values of "free cysteine" for normal adult rabbit liver (table 2) to values of glutathione in milligrams per 100 gm. of tissue. The result is 296.4. Yamamoto (*21*) reported 315.8 mg. glutathione per 100 gm. of rabbit liver tissue.

Whether the material titrated in the deproteinized liver filtrates and in the crude, untreated tissue extracts is glutathione or not, it is nevertheless certain that there is present in these fluids some substance or substances which react with nitroprusside to give a color characteristic of mercaptans, and that when certain oxidants are progressively added to these fluids the intensity of the nitroprusside reaction steadily decreases until a concentration of the oxidant is reached at which the latter reaction disappears. Apparently nearly the same amount of oxidation is required in the deproteinized fluids as in the crude extract to reach such an end point, as is shown by the independent investigators mentioned (*17*, *18*, *19*, *20*, *21*). The mercaptan substance or substances could be glutathione, cysteine, hydrogen sulfide; in the case of the crude extract, the proteins might contribute sulfhydryl groups.

[1] *Thompson and Voegtlin reported the use of nitroprusside as an external indicator in the titration of sulfhydryl groups many years before it was applied to denatured proteins.*

472192—42——5

Voegtlin and Thompson (*22*) reported a negative Sullivan reaction for cysteine and cystine in their tissue extracts. Hydrogen sulfide in any appreciable quantities may be ruled out because the addition of lead, copper, or silver salts to the deproteinized filtrates does not result in any evidence of blackening. Although no direct evidence is yet available that all of the mercaptan in these fluids is glutathione, it seems probable that it is either glutathione or some as yet unknown sulfhydryl-containing substance or substances nonprecipitable by protein reagents.

The greater value for the sulfhydryl group in rabbit liver than for that found in rat liver (table 2) is consistent with the earlier work of Greenstein and Jenrette (*23*) on the nucleoprotein fraction of the livers of animals of several species. The relative values for the mercaptan groups in these species is also illustrated by the higher values found by Thompson and Voegtlin (*17*) for the glutathione content of the red cells of the rabbit as compared with that of the rat.

The sulfhydryl-group values of regenerating rat liver and of the livers of tumor-bearing rats appear to be the same as the value for normal rat liver (table 2). The necrotic portion of the hepatoma 31 transplants is nearly devoid of sulfhydryl groups, a result consistent with that reported for the necrotic areas of other tumors by Voegtlin and Thompson (*22*).

The behavior of extracts of normal mouse livers and mouse hepatomas towards guanidine hydrochloride is similar to that of the corresponding rat tissues, i. e., there is an increase in the number of sulfhydryl groups found in the extract after addition of the salt. The extent of this increase is, however, significantly greater in the mouse tissues than in those of the rat. Furthermore, there is apparently no difference in the sulfhydryl content of the normal mouse livers, the livers of tumor-bearing mice, and the different kinds of transplanted hepatomas (table 2). This illustrates another interesting difference between rat and mouse hepatomas (*24*).

The content of sulfhydryl groups in the fetal rabbit livers is extremely low (table 2). The activity of many of the enzyme systems in the fetal liver is also very much lower than that of adult rabbit liver (*24*).

## SUMMARY

The sulfhydryl groups of aqueous extracts of normal and tumorous hepatic tissues were measured before and after the addition of a wide variety of salts. The tissues included normal rat, mouse, and rabbit liver, transplants of rat hepatoma 31, mouse hepatomas 587, 98/15, and A, regenerating rat liver, the livers of tumor-bearing rats and mice, and fetal rabbit livers. Different times of extraction and different concentrations of salt were employed.

The values for the sulfhydryl groups of the untreated extracts of rat and of rabbit liver correspond with those reported for glutathione by several investigators.

With the exception of phosphate and the ammonium salts, all salts when added to the aqueous extracts of the hepatic tissues studied caused an increase in the number of sulfhydryl groups titrated. This has been interpreted as a denaturation of the extracted proteins. Phosphate and the ammonium salts did not cause any change from the values found in the untreated extracts.

The extent of the denaturation by salts in rat liver tissue is much greater than that in the rat hepatoma. In mice, the values of the hepatoma and of normal liver extracts after denaturation are the same for the various tumors and for different strains.

The sulfhydryl content of the rabbit liver is much greater than that of the rat. The content of mercaptan groups in the necrotic area of the hepatoma 31 transplants and in fetal rabbit liver is extremely small. The sulfhydryl content of regenerating rat liver is the same as that of normal rat liver. Livers of the tumor-bearing animals have the same mercaptan content as the livers of normal animals.

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# Genetic Analysis of Susceptibility to Induced Pulmonary Tumors in Mice

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This genetic analysis of susceptibility to induced pulmonary tumors takes into consideration not only tumor incidence but also the time at which the tumors arose and the number of nodules found in each individual.

Recent work (*1–4*) has indicated that susceptibility to pulmonary tumors is not a character with definite alternative expression, that the inbred strains of mice and even the individuals themselves cannot be grouped definitely in the two classes, susceptible and resistant, but that susceptibility to this neoplasm is a character expressed in degree. In these previous experiments it was found that mice varied in the time required for the tumors to develop, and this caused the tumor incidence to be dependent upon the time at which the observation was made. With induced pulmonary tumors it appeared that in general those strains considered as susceptible on the basis of tumor incidence developed their tumors early, whereas the resistant strains developed theirs later. Furthermore, the animals varied as to the number of nodules which appeared. In certain mice only 1 nodule developed while in others as many as 100 or more induced nodules were found. Here again strains generally considered as susceptible developed many nodules, whereas resistant strains developed fewer nodules.

It was thus apparent that to analyze satisfactorily the inheritance of the character, this variation in degree of susceptibility had to be taken into consideration. In the experiment reported here an attempt was made to do this by these two suggested methods: (1) By determining the time at which the tumors appeared; and (2) by counting the nodules that had developed.

## EXPERIMENTAL PROCEDURE

The two parent inbred strains used in this work were strain A mice which are highly susceptible to the development of pulmonary tumors, and strain L mice which are extremely resistant.[1] Strain A mice were mated to strain L to produce the $F_1$ hybrid generation. The $F_1$ hybrids were mated to $F_1$ to produce the $F_2$ hybrid generation, and the $F_1$ hybrids were backcrossed to each parent strain to produce both types of backcross generations.

A total of 1,520 mice, including the 2 inbred strains and the 4 hybrid groups, were tested for susceptibility. At 8 weeks (56 days) of age, each animal was injected intravenously with 0.5 mg. 1,2,5,6-dibenzanthracene dispersed in 0.5 cc. horse serum.[2] Approximately 1 l. of this dispersion was prepared at the beginning of the work, and this sufficed for the whole experiment, thus eliminating any possible variation due to differences in preparation of the dispersion.

To get the complete picture of the susceptibility of each strain and hybrid generation, sample groups were killed at regular intervals and the number of

[1] *The strain A mice were derived from Dr. J. J. Bittner's strain A colony at the Roscoe B. Jackson Memorial Laboratory and the strain L mice were derived from Dr. A. M. Cloudman's strain L colony at the same laboratory.*

[2] *Dr. Egon Lorenz, of the National Cancer Institute, prepared the dispersion.*

animals with tumors was noted and also the number of nodules appearing on the surface of the lungs of each individual was recorded. At the outset it was planned that with each parent strain and hybrid generation one group would be killed at approximately the time the first tumors appeared and this group would be followed at monthly intervals by additional groups until the 100-percent-tumor incidence had been reached. This was designed to give complete information on the time at which the tumors arose. For the most part this procedure was followed, but as the experiment progressed the plan was somewhat altered to reveal certain important information as will be pointed out later. In the strain L, the $F_1 \times$ strain L backcross hybrids, and in the $F_2$ hybrids, the 100-percent-tumor incidence was not reached.

This observation of the tumors and the counting of the nodules were done with the fresh lung placed under the dissecting microscope. While it would have been impractical to have sectioned every nodule, the diagnoses of those of all doubtful individuals were checked with histologic sections.

An attempt was made to maintain all animals under identical environmental conditions. They were all fed Purina dog chow and were given an unlimited supply of tap water.

Approximately 3-percent mortality was experienced at the time of the injection, and in the latter weeks of the experiment some of the strain L mice became heavily infested with the mite *Myobia musculi*, which caused them to become badly mutilated from scratching, and some of these animals died and others were discarded. The only other mice that died were a few which were accidentally killed. The data presented are of effective totals.

## RESULTS

The data of the experiment in complete form are presented in table 1. The tumor incidence of each group is given, and, furthermore, each animal is tabulated according to its number of tumor nodules. Mean number of nodules and standard deviation were calculated on the actual counts rather than the grouped data presented in the table. In view of the fact that in no group was there found to be a significant sex difference and also since previous work has failed to demonstrate a sex difference in susceptibility to pulmonary tumors, the data for both sexes are combined.

The strain A mice developed their tumors early. Five of the thirty mice killed 4 weeks after the injection had tumors, although each animal had but one nodule. Twenty-eight strain A mice which had originally been scheduled with the 4-week group were killed at 6 weeks following the injection, and 100 percent of this group had tumors. Also each of the three subsequent strain A groups killed at 8, 12, and 16 weeks showed a tumor incidence of 100 percent.

Not only did the tumors arise early in the strain A mice but many nodules developed. The 5 strain A mice with tumors at 4 weeks each had 1 nodule making a mean number of 0.16 for the group; but as the animals were permitted to live longer, the number of nodules per individual increased, reaching a mean of 75.43 at 16 weeks. In each group the variation of the number of nodules was determined and represented by the standard deviation. It was found that as the mean number of nodules increased the standard deviation also increased as is typical of biologic data of this kind.

In contrast with strain A, the resistant strain L mice developed their tumors very late, and seldom was more than 1 nodule

found in an individual. The earliest appearance of any tumor was 28 weeks after the injection. At that time 1 mouse of 41 had developed a small tumor. The tumor incidence rose slowly in this strain, reaching only 24 percent by 48 weeks. In only 2 cases did strain L mice display more than 1 nodule, and those 2 animals had but 2 nodules each. All of these strain L tumors and also the doubtful

TABLE 1.—*Development of pulmonary tumors in strain A and strain L mice, and their $F_1$, $F_2$, and backcross hybrids injected intravenously at 56 days of age with 0.5 mg. 1, 2, 5, 6-dibenzanthracene*

| Time after injection (weeks) | Number of mice | Mice showing given number of tumors per individual | | | | | | | | | | | | | | | | | | | | | | | Mice with tumors | Mean number of nodules [1] | Standard deviation [1] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1–5 | 6–10 | 11–15 | 16–20 | 21–25 | 26–30 | 31–35 | 36–40 | 41–45 | 46–50 | 51–55 | 56–60 | 61–65 | 66–70 | 71–75 | 76–80 | 81–85 | 86–90 | 91–95 | 96–100 | 101–105 | 106–110 | 111–115 | | | |
| **STRAIN A** | | | | | | | | | | | | | | | | | | | | | | | | | *Percent* | | |
| 4 | 30 | 25 | 5 | | | | | | | | | | | | | | | | | | | | | | 17 | 0.16 | 0.22 |
| 6 | 28 | | 13 | 8 | 5 | 1 | | 1 | | | | | | | | | | | | | | | | | 100 | 7.75 | 5.90 |
| 8 | 51 | | | 4 | 13 | 15 | 6 | 4 | 5 | 3 | | | 1 | | | | | | | | | | | | 100 | 21.00 | 9.30 |
| 12 | 10 | | | | | | | | | | | | 1 | 1 | 3 | | 3 | | | | 1 | 1 | | | 100 | 71.10 | 14.30 |
| 16 | 108 | | | | | | | | | 1 | 3 | 2 | 5 | 4 | 17 | 12 | 12 | 11 | 12 | 9 | 9 | 3 | 6 | 1 | 1 | 100 | 75.43 | 15.74 |
| **STRAIN L** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 24 | 22 | 22 | | | | | | | | | | | | | | | | | | | | | | | 0 | | |
| 28 | 41 | 40 | 1 | | | | | | | | | | | | | | | | | | | | | | 2 | 0.02 | |
| 32 | 68 | 63 | 5 | | | | | | | | | | | | | | | | | | | | | | 7 | .07 | |
| 40 | 77 | 68 | 9 | | | | | | | | | | | | | | | | | | | | | | 12 | .13 | |
| 48 | 46 | 35 | 11 | | | | | | | | | | | | | | | | | | | | | | 24 | .27 | |
| **$F_1$ HYBRIDS** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | 50 | 47 | 3 | | | | | | | | | | | | | | | | | | | | | | 6 | 0.12 | 0.47 |
| 8 | 51 | 37 | 14 | | | | | | | | | | | | | | | | | | | | | | 27 | .41 | .80 |
| 10 | 50 | 11 | 38 | 1 | | | | | | | | | | | | | | | | | | | | | 78 | 1.66 | 1.35 |
| 12 | 37 | | 27 | 9 | 1 | | | | | | | | | | | | | | | | | | | | 100 | 4.16 | 2.58 |
| 16 | 103 | | 5 | 37 | 39 | 15 | 5 | 1 | | 1 | | | | | | | | | | | | | | | 100 | 12.46 | 5.26 |
| **$F_2$ HYBRIDS** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 25 | 25 | | | | | | | | | | | | | | | | | | | | | | | 0 | | |
| 6 | 20 | 19 | 1 | | | | | | | | | | | | | | | | | | | | | | 5 | 0.05 | |
| 8 | 81 | 57 | 21 | 2 | | 1 | | | | | | | | | | | | | | | | | | | 30 | .95 | 2.61 |
| 12 | 99 | 44 | 31 | 10 | 6 | 3 | 3 | 1 | | 1 | | | | | | | | | | | | | | | 56 | 4.43 | 7.11 |
| 16 | 105 | 26 | 31 | 13 | 11 | 5 | 8 | 2 | 3 | 2 | 1 | 1 | | | | 1 | | 1 | | | | | | | 75 | 9.96 | 14.07 |
| 20 | 101 | 14 | 29 | 15 | 12 | 8 | 4 | 4 | 5 | 5 | 2 | | | | | 3 | | | | | | | | | 86 | 13.21 | 15.12 |
| 24 | 100 | 6 | 28 | 7 | 10 | 8 | 6 | 7 | 10 | 4 | 6 | | 2 | 3 | 1 | | 2 | | | | | | | | 94 | 19.56 | 18.09 |
| **A-BACKCROSS HYBRIDS** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | 29 | 17 | 11 | | 1 | | | | | | | | | | | | | | | | | | | | 41 | 1.10 | 2.16 |
| 8 | 21 | 4 | 10 | 4 | | 1 | | 1 | 1 | | | | | | | | | | | | | | | | 81 | 6.38 | 8.61 |
| 10 | 20 | | | 4 | 2 | 2 | | 2 | 3 | 1 | 2 | 1 | 2 | | 1 | | | | | | | | | | 100 | 29.40 | 16.62 |
| 16 | 41 | | | | 2 | 8 | 3 | 2 | 7 | 3 | | 4 | | 3 | 3 | 2 | | 1 | | 1 | | | 1 | | 1 | 100 | 41.90 | 24.28 |
| **L-BACKCROSS HYBRIDS** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 12 | 12 | 12 | | | | | | | | | | | | | | | | | | | | | | | 0 | | |
| 16 | 30 | 18 | 11 | 1 | | | | | | | | | | | | | | | | | | | | | 40 | .73 | 1.28 |
| 24 | 18 | 7 | 4 | 5 | 1 | | | 1 | | | | | | | | | | | | | | | | | 61 | 5.00 | 7.15 |
| 32 | 18 | 4 | 6 | 2 | 1 | 2 | 1 | 2 | | | | | | | | | | | | | | | | | 78 | 8.66 | 9.85 |
| 40 | 15 | 5 | 6 | | 2 | 1 | | | | | 1 | | | | | | | | | | | | | | 67 | 6.40 | 11.86 |

[1] Calculated from the raw data.

tumors of the other groups were diagnosed with the histologic sections. While for the most part they were small nodules, all listed as tumors were classified as pulmonary tumors by the pathology group of the National Cancer Institute. Six strain L mice had nodules so small that they could not be definitely classified as tumors, and these animals were listed as nontumorous.

Three $F_1$ hybrids out of the fifty killed at 6 weeks had tumors, and through the subsequent groups the tumor incidence rose, reaching 100 percent at 12 weeks. Again, like the strain A mice, as the animals were given a longer time in which to develop tumors, the mean number of nodules increased with a corresponding increase in standard deviation.

In previous work no difference was reported in susceptibility between reciprocal hybrids, and in this work no significant difference was found in the 8-week and 12-week $F_1$ groups. A significant difference was observed, however, in the 16-week group. In this series, the $F_1$ hybrids from strain L (resistant) mothers had a slightly higher mean number of nodules than did those from strain A (susceptible) mothers, the difference being statistically significant. Since this difference occurred in but one experiment, its interpretation must be withheld until further experimentation has been carried on. It is interesting to note, however, that the difference was due primarily to a difference in the males, which in itself is suggestive of Y-chromosome inheritance.

The results of the $F_2$ hybrids were particularly interesting. Tumors began to appear 6 weeks after the injection, and the incidence showed a gradual rise through subsequent periods, reaching as high as 94 percent at 24 weeks. As time increased, the mean number of nodules became greater with an accompanying increase in standard deviation. These results, however, did not parallel those of the strain A and $F_1$ groups, as is shown by the analysis of this variability.

The number of backcross animals tested was somewhat less than the number of $F_2$ hybrids. Nevertheless, the results obtained from the backcross generations are significant. The A-backcross hybrids had an incidence of 41 percent at 6 weeks and 100 percent at 10 weeks; while tumors were first observed in the L-backcross hybrids at 16 weeks, and 100-percent incidence had not been reached by 40 weeks. In both generations with increase in time the mean number of nodules and variation in number showed a gradual increase with the exception of the group of L-backcross hybrids killed at 40 weeks. This group showed a lower tumor incidence and also a lower mean number of nodules than did the group killed at 32 weeks, although there was an increase in the standard deviation. However, there were few animals in this group, and the results fell within the limits of chance variation.

## ANALYSIS ON THE BASIS OF LATENT PERIOD

From the foregoing data an analysis of the inheritance of induced pulmonary tumors was made, by measuring susceptibility on the basis of the time required for the tumors to develop following the injection. The susceptibility of each parent strain and each of the four hybrid groups is represented in figure 1. Points were located by plotting tumor incidence against time for each group, after which these points were connected by lines, thus forming the curves shown.

On this basis the strain A mice could be classified as extremely susceptible, 17 percent having developed tumors as early as 4 weeks and 100 percent having

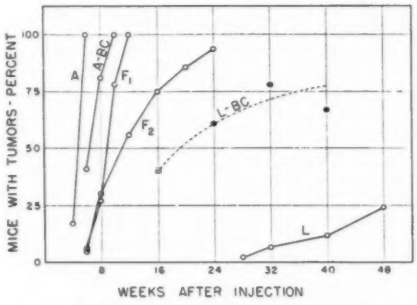


FIGURE 1.—Tumor incidence in strain A and strain L mice and their $F_1$ and $F_2$ and backcross hybrids killed at various intervals following the intravenous injection of 0.5 mg. 1, 2, 5, 6-dibenzanthracene. Solid circles: Groups of less than 20 mice.

tumors by 6 weeks. On the other hand the strain L mice were extremely resistant. No tumors were found before 28 weeks after the injection, and an incidence of only 24 percent was reached by 48 weeks. The $F_1$ generation mice were intermediate to the parent strains in their susceptibility but tended to approach the strain A. The $F_2$ mice were likewise intermediate to the two parent strains. When the $F_1$ hybrids were backcrossed to the susceptible strain A, an A-backcross generation was produced which was intermediate to the $F_1$ and the strain A. Likewise when the $F_1$ hybrids were backcrossed to the resistant parent strain L, the L-backcross generation produced was intermediate to the $F_1$ and strain L.

While the position of these hybrid groups is indicative of genic action, the most striking evidence that genetic factors were involved was the increase in variability of the $F_2$ generation, in which one could have expected variation due to segregation of genetic factors over that of the $F_1$ generation which was genetically homogeneous. This can be observed in figure 1 where variability is represented by the spread along the abscissa. Tumors began to appear in the $F_1$ generation at 6 weeks and 100 percent was reached at 12 weeks, whereas in the $F_2$ generation, while tumors began to appear also at 6 weeks and while the tumor incidence showed a gradual increase, 100 percent was not reached in the course of the experiment. The incidence in a group of 100 animals killed at 24 weeks was 94 percent, and of a small group of 13 $F_2$ mice kept until 28 weeks 1 showed no tumors, which indicated that 100 percent could not be expected until after 28 weeks. Thus, the $F_2$-generation hybrids varied more in their susceptibility than did those of the $F_1$ when susceptibility was measured by latent periods.

There was evidence that a part of this increase in variability of the $F_2$ over that of the $F_1$ generation may have been due to an increase in nongenetic variation. There is an indication that as the latent period was increased, which gave more time for nongenetic effects, the variation caused by these nongenetic factors increased. Strain L mice, which had a much greater latent period than did strain A mice, were much more variable; and since both were isogenic strains, this increase in variability must have resulted from the action of nongenetic factors. However, even with this in mind, the expected increase in nongenetic variability of the $F_2$ over that of the $F_1$ generation would be slight. It seems evident that the mean latent period of the $F_2$ is but little greater than that of the $F_1$, and, furthermore, the distribution of the $F_2$ generation completely overlaps that of the $F_1$. It is thus safe to assume that at least a part of this increased variability of the $F_2$ generation was due to the segregation of genetic factors.

The results as a whole, with the respective positions of the hybrid groups and

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with the increase in variability of the $F_2$ over that of the $F_1$, are typical of those of the host of inherited quantitative characters that have been analyzed in the field of animal genetics and especially in the field of plant genetics. The interpretation of such results can be only that the character is inherited on a multiple-factor basis.

Furthermore, there is no evidence that any one factor is a major factor, with the others acting as modifiers. The $F_2$ generation shows all gradations in degree of susceptibility between the two parent strains, and there is no indication of bimodality as would be expected with either a dominant or recessive single major factor or that the curve is a trimodal curve as would be expected with a major factor expressing no dominance.

## ANALYSIS ON THE BASIS OF NUMBER OF NODULES

The picture obtained by measuring susceptibility on the basis of number of tumor nodules per individual was strikingly similar to that on the basis of latent period. Here again the $F_1$ and $F_2$ generations showed susceptibility intermediate to the two parent strains. The A-backcross generation was intermediate to the $F_1$ and the parent strain A, and the L-backcross was intermediate to the $F_1$ and strain L. This was best represented by the groups which were killed at 16 weeks after the injection and from which the frequency polygons in figure 2 were constructed.

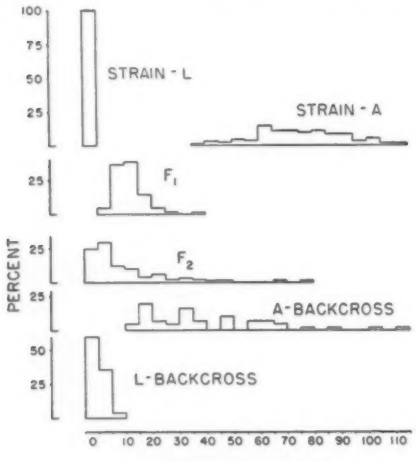


FIGURE 2.—Frequency polygons for number of tumor nodules in the parent strains A and L, and their $F_1$, $F_2$, and backcross hybrids at 16 weeks after the intravenous injection of 0.5 mg. 1, 2, 5, 6-dibenzanthracene.

The 16-week groups were chosen for constructing the frequency polygons since it was desirous to keep the time factor uniform, and it would have been impractical to have attempted to count the nodules in a strain A group kept for a longer time. Yet more symmetrical polygons would have been obtained from the data of some of the hybrid groups killed at later periods. The polygons of all the hybrid groups killed at 16 weeks tend to be skewed toward the parent strain L. This, however, should not be interpreted as meaning that there was a general tendency for the degree of susceptibility of these hybrid groups to fall in the direction of the parent strain L. Had the hybrids been kept until some later time at which the strain L mice had developed tumors, this skewness would probably have completely disappeared as is suggested especially by the later groups of $F_2$ hybrids.

It should be pointed out that the picture presented by these frequency polygons is what one could well expect to get in analyzing an inherited quantitative character controlled by multiple factors.

In the strain A mice and in each of the four hybrid generations it was found that as the mean number of nodules increased, the variation in number of nodules also increased. This has been represented graphically by plotting standard devia-

tion against the square root of the mean [3] (fig. 3).

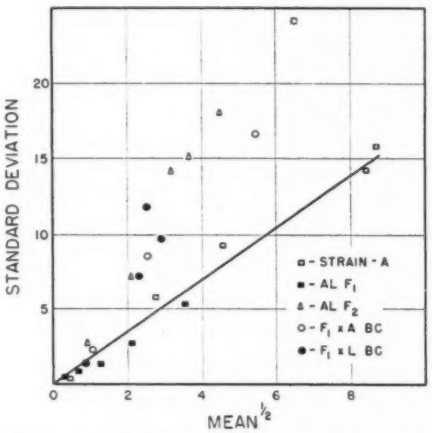


FIGURE 3.—The square root of the mean number of lung nodules plotted against the standard deviation.

Since both the strain A and strain L mice had been inbred by brother-to-sister matings for well over 50 generations and each represented but a single subline, it is safe to assume for practical purposes that no genetic variation would have occurred in either strain. Furthermore, the $F_1$ generation also would have been genetically homogeneous. Thus, the line drawn through the $F_1$ and strain A points would represent the nongenetic variation that could have been expected for any group of animals with a given mean within the limits of the experiment. It is interesting to note that this variation due to nongenetic factors was considerable.

After having located this line, it was easy to demonstrate that genetic factors were involved. If such factors were involved, one would expect that owing to the segregation of these factors the variation of the $F_2$ and backcross hybrids would have been greater than that found in the genetically homogeneous groups. When the $F_2$ points were plotted, it was found that they tended to fall in a line representing much more variation than that which could be attributed to nongenetic factors alone. Furthermore, the points of the two types of backcross hybrids likewise tended to fall in a line representing greater variation than that of the genetically homogeneous group; but with the exception of the one point, the variation was not so great as that of the $F_2$ groups. This also is in accord with multiple-factor inheritance.

From these data obtained by measuring susceptibility on the basis of number of tumor nodules, it was possible to obtain an estimate of the relative value of genetic and environmental factors in producing the variance of the $F_2$ generation. By considering the nongenetic variance of the $F_2$ as measured by the variance of the $F_1$ and thus the genetic variance of the $F_2$ as the difference between the total variance of the $F_2$ and that of the $F_1$, it was estimated that nongenetic factors accounted for approximately 14 percent of the total variance of the $F_2$ generation, and genetic factors for 86 percent. The data for the 16-week groups were applied, since they were more extensive than those of the other groups and would, therefore, probably give a more accurate estimate than would the data for the other groups.

## ESTIMATION OF NUMBER OF GENETIC FACTORS

From these data, obtained by measuring susceptibility on the basis of number of nodules per individual, an estimate of the least possible number of genetic factors involved could be made. In 1921 Wright (*5*) and Castle (*6*) presented a formula which was later elaborated by Serebrovsky (*7*) and Bernstein (*8*) for estimating the number of genetic factors involved

[3] *This transformation was used solely for convenience in plotting the information and is not necessarily intended to represent the nature of the relationship between an increase in the mean number of nodules and the variability.*

in inheritance of this kind. This formula, as presented in one of the more simple forms, was

$$\eta = \frac{\Delta^2}{8(\sigma_{F_2}^2 - \sigma_{F_1}^2)}$$

i. e., the number of pairs of genes involved is equal to the square of the difference between the parental strains divided by 8 times the genetic variance of the $F_2$ generation. When the backcross generation is employed, the genetic variation is multiplied by 16.

By applying the formula to the data for the 16-week groups and using the variance of the $F_2$ generation, the minimum number of genetic factors was estimated as four pairs. The difference between the two parent strains was taken as the mean number of nodules found in the strain A mice at 16 weeks, since at that period no nodules had developed in the strain L animals. Could this difference have been determined at a later time when the strain L mice had developed tumors, it is obvious that the difference between the two parent strains would have been greater, and it is highly probable that the increase would have been great enough to have made an increase in the estimate of the number of genetic factors. It should also be pointed out that any possible degree of dominance or epistasis would tend to increase the estimate. However, it is safe to state that the least possible number of genetic factors influencing susceptibility by which strain A and strain L differ is four pairs, but there are probably more.

## DISCUSSION

For some time it has been generally accepted that susceptibility to pulmonary tumors in mice is influenced by genetic as well as environmental factors, yet conclusions as to the number and action of these factors have not been wholly in accord. As early as 1926 Lynch (*9*) showed that heredity influenced the tendency to form tumors of the lung and concluded that the character was dominant in nature, although she recognized that the character was extremely variable. Later from rather extensive data on strain C57 Black, strain A, and their $F_1$ and $F_2$ hybrids, Bittner (*10*) obtained ratios which suggested that but a single dominant factor was involved, and following this Andervont (*11*) published backcross data for induced pulmonary tumors which tended to substantiate Bittner's conclusions. Work which has followed, however, has shown that the situation is not so simple.

The author (*1*) found that when strain A mice were outcrossed to three other inbred strains the three groups of $F_1$ hybrids produced differed in their degree of susceptibility. Furthermore, especially when the number of nodules was considered, the $F_1$ hybrids tended to be intermediate to their parent strains. This suggested the action of multiple factors, as was likewise suggested by the difference in the time of development of the nodules. Andervont (*12*) also recently published data which suggested that more than one factor was involved, but whether or not the action was one of a single major factor with modifying factors was not indicated. Lynch (*3*) showed, from her data obtained by counting the nodules in each individual, that the degree of susceptibility as measured by the number of nodules is affected by heredity but she did not determine the number of factors involved.

The situation was clarified by integrating these various factors, tumor incidence, latent period, and number of nodules, into the one major project reported here. While the shortcomings of any analysis on the basis of tumor incidence alone were obvious because of the variation in degree of susceptibility, it was shown that the

degree of susceptibility could be adequately measured by the latent period and also by the number of nodules. The most obvious proof of this was the manner in which the results of the two methods tended to parallel.

The measuring of the degree of susceptibility carried the analysis into the realm of quantitative characters, making possible the application of methods similar to those commonly employed in analyzing such characters. Although these quantitative methods for the most part had not been previously employed in the tumor field, the results in this work were encouraging and they revealed the prospects of further application of such methods to the analysis of the inheritance of other types of neoplasms.

The increase of variability of the time at which the $F_2$ mice developed tumors over that at which the $F_1$ mice did, along with the increase of variability of the number of nodules in the animals of the $F_2$ and backcross generations over the $F_1$ and strain A, soundly confirmed the conclusion that genetic factors were involved. Furthermore, it was clearly shown that these genetic factors were multiple in number. The degree of susceptibility of the $F_1$ and of the $F_2$ hybrids as measured by both methods fell intermediate to the parent strains. By both methods the A-backcross hybrids were found to fall between the $F_1$ and the parent strain A, while the L-backcross fell between the $F_1$ and the parent strain L. The $F_2$ hybrids displayed no clear-cut segregation into definite classes but showed all degrees of susceptibility between the two parent strains. In a somewhat less definite manner the backcross generations showed continuous gradations between the $F_1$ and the respective parent inbred strain.

The possibility of a single major factor with modifying factors was ruled out by the unimodality of the $F_2$ curve.

As has been true in general with quantitative characters, the exact number of genetic factors involved was not determined. By applying the formula to the data on the number of nodules, however, a rough estimate of the least possible number of genetic factors by which these two strains differed was obtained. This was four pairs, but it was pointed out that the number was probably greater than this estimate.

This does not indicate that all inbred strains with a high susceptibility to pulmonary tumors should differ from all resistant strains by the same number of factors, and there is no reason to suspect that they would. The more logical assumption would be that the strains generally classified as high-pulmonary-tumor strains may differ in their respective number of genetic factors influencing susceptibility, and the same is probably true of the strains classified as resistant. That different tumor incidences have been obtained in different $F_1$ generations resulting from crossing various inbred strains supports this assumption. Thus, it is possible that the strain A and strain C57 Black do differ by but one dominant factor for susceptibility as reported by Bittner. The results presented here, however, heighten the skepticism of such a simple interpretation and stress the need of breeding tests of $F_2$ or backcross segregants for verification. This is especially true in view of the fact that the mean age of his strain A mice was 19.5 months for the females and 16.1 months for the males, whereas that of the $F_1$ hybrids was as much as 26.7 months and that of the $F_2$ hybrids was 23.7 months.

Linkage tests previously reported by the author (*13*) showed that susceptibility to induced pulmonary tumors was associated with the chromosome bearing the shaker-2 and waved-2 gene loci and also that bearing the flexed-tail gene locus. These find-

ings, which indicate that susceptibility factors are on these two different chromosomes, are in accord with the multiple-factor interpretation.

Again the role of environmental factors should be mentioned. It has been pointed out repeatedly that environmental factors do influence susceptibility to pulmonary tumors, but heretofore no indication of the relative weight of such factors as compared with genetic factors has been given. In this experiment it was estimated that of the total variance of the $F_2$ generation approximately 86 percent was due to genetic factors and the remaining 14 percent was attributed to nongenetic factors.

## SUMMARY

The present work has shown and confirmed that susceptibility to induced pulmonary tumors in mice is not a character with alternative (all-or-none) expression, but that susceptibility is expressed in degree. This degree of susceptibility can be adequately measured quantitatively in two ways (1) by the time required for the tumors to develop, and (2) by the number of nodules that develop.

The results obtained by the two methods for measuring susceptibility are strikingly parallel. The results of both methods confirm the conclusion that genetic factors as well as environmental factors are involved. It is also clearly shown by both methods that the genetic factors influencing susceptibility are multiple in number.

There is evidence in opposition to the possibility that there is one major factor with modifying factors.

The rough estimate of the minimum number of susceptibility factors by which strain A and strain L differ is four pairs, and it is pointed out that there are probably more.

Of the total variance in susceptibility of the $F_2$ generation, as measured by the number of tumor nodules, it is estimated that approximately 86 percent is due to genetic factors and 14 percent to nongenetic factors.

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# Inheritance of Susceptibility to Spontaneous Pulmonary Tumors in Mice

By W. E. Heston, *geneticist, National Cancer Institute, National Institute of Health, United States Public Health Service*

This paper presents data on the genetics of spontaneous pulmonary tumors in mice and points out how they tend to fall in line with the conclusions previously drawn for the inheritance of induced pulmonary tumors.

It has been conclusively shown that susceptibility to induced pulmonary tumors in mice is inherited on a multiple-factor basis. In preliminary experiments published by the author in 1940 (*1*), it was found that when the susceptible strain A mice were outcrossed to mice of three resistant strains, the $F_1$ hybrids produced in each case were intermediate in degree of susceptibility between the respective parent strains. Furthermore, the three $F_1$ hybrid groups differed in their degree of susceptibility. These results suggested multiple-factor inheritance as did others published by Andervont (*2*). Later by an extensive analysis which took into consideration not only the tumor incidence but also the time at which the tumors arose and the number of nodules per individual, it was definitely shown that multiple factors were involved (*3*).

In view of the results of the preliminary experiments, it seemed advisable to obtain data for spontaneous pulmonary tumors to see whether they would parallel those for induced tumors. The results of the induced tumors were not in accord with the single-dominant-factor mode of inheritance which Bittner had proposed earlier for spontaneous pulmonary tumors (*4*). In strains A and C57 Black and their $F_1$ and $F_2$ hybrids, Bittner had observed tumor incidences which could have been expected if susceptibility had been controlled by a single dominant factor. It should be noted, however, that his $F_1$ and $F_2$ hybrid mice had been autopsied at an average age which was much greater than that at which the strain A mice were. The work with induced tumors strongly emphasized that the incidence of induced tumors was dependent upon the time at which the observation was made, and thus it seemed desirable to obtain further data on spontaneous tumors, keeping the age factor constant, and to see whether or not they tended to substantiate Bittner's earlier conclusions or tended to fall in line with the results of the induced tumors.

## EXPERIMENTAL PROCEDURE

Strain A, which has a high degree of susceptibility to spontaneous pulmonary tumors, and strain L, which has a high degree of resistance, were used as the parent strains.[1] From these two strains, $F_1$ hybrids and also backcross hybrids resulting from mating the $F_1$ to the parent strain L were produced.

These strain A and strain L mice were of the same stocks as those employed in the previous induced-tumor work. Furthermore, the hybrids of this experiment resulted from matings which had also been used to produce the test hybrids of the induced-tumor experiments. Thus, for the most part the mice yielding the spontaneous tumor data presented here were siblings of those tested for induced tumors, in the work published earlier.

[1] *See preceding paper* (3) *for the origin of strains.*

Groups of mice of the two inbred strains and the two types of hybrids were produced and set aside for later observation of the spontaneous-tumor incidence, an attempt being made to rear them all under identical conditions. They were maintained in wooden cages in lots of eight animals to the cage, were fed Purina dog chow, and were given an unlimited supply of tap water. The sexes were kept segregated.

The tumor-incidence data were gathered at definite age periods. Sample groups of strain A mice and of the $F_1$ hybrids were killed at three age levels, 12, 15, and 18 months. The strain L mice and the backcross hybrids, however, were killed at only the 15- and 18-month periods. Although some deaths occurred, the data presented here are only from those mice which lived to the definite age periods and were killed at those periods.

At autopsy the lungs were removed and placed under the dissecting microscope for the observation of the tumors. Not only the presence of tumors but also the number of nodules per individual was recorded.

## RESULTS AND DISCUSSION

The tumor incidence in the group of strain A mice killed at 12 months was 53 percent, but the incidence had increased to 79 percent in the group killed at 15 months and was as high as 90 percent in the group killed at 18 months (table 1). It should be noted that these percentages are comparable with those published earlier by Bittner (*1*). He observed 74.4 percent in strain A males at an average age of 16.1 months, and 89.2 percent at an average age of 19.5 months.

No pulmonary tumors were found in the strain L mice killed at 15 months and also none in those killed at 18 months. This, however, could have been expected from past experience with strain L mice. Cloudman [2] has stated that he has found only two spontaneous pulmonary tumors in his strain L colony at the Roscoe B. Jackson Memorial Laboratory. In our colony of strain L mice only one spontaneous pulmonary tumor has been observed.

TABLE 1.—*Incidence of spontaneous pulmonary tumors in strain A and strain L mice, and their $F_1$ and L-backcross hybrids killed at 12, 15, and 18 months of age*

| Type of mice | Age | Number | Tumor incidence | Mice showing given number of nodules per individual | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 0 | 1 | 2 | 3 | 4 |
| | *Months* | | *Percent* | | | | | |
| Strain A | 12 | 32 | 53 | 15 | 13 | 4 | .... | .... |
| $F_1$ hybrids | 12 | 32 | 31 | 22 | 10 | .... | .... | .... |
| Strain A | 15 | 92 | 79 | 19 | 44 | 18 | 8 | 3 |
| $F_1$ hybrids | 15 | 60 | 48 | 31 | 23 | 5 | 1 | .... |
| L-backcross | 15 | 99 | 15 | 84 | 11 | 4 | .... | .... |
| Strain L | 15 | 51 | 0 | 51 | .... | .... | .... | .... |
| Strain A | 18 | 50 | 90 | 5 | 24 | 13 | 6 | 2 |
| $F_1$ hybrids | 18 | 55 | 53 | 26 | 18 | 10 | .... | 1 |
| L-backcross | 18 | 93 | 22 | 73 | 16 | 4 | .... | .... |
| Strain L | 18 | 50 | 0 | 50 | .... | .... | .... | .... |

It can be readily seen from the data that the $F_1$ hybrid mice resulting from outcrossing the strain A to the strain L were not so highly susceptible as were the parent strain A mice. To compare with the three percentages for the strain A mice at the definite age periods, the $F_1$ hybrids gave incidences of only 31 percent at 12 months, 48 percent at 15 months, and 53 percent at 18 months. Whether or not at a much greater age these $F_1$ hybrids would have had a tumor incidence as high as that of the strain A mice at 18 months is problematic. It is obvious, however, that at a comparable age the tumor incidence of the $F_1$ hybrids was not so great as that of the parent strain A. Statistically, the difference between the 12 months groups was border line in significance, $\chi^2=3.14$; $P<10$ percent. In these two groups, however, the numbers were not large. The difference

[2] *Personal communication.*

between the 15-months groups was statistically significant, $\chi^2=15.9$; $P<0.01$ as was also the difference between the 18-months groups, $\chi^2=19.8$; $P<0.01$.

The backcross hybrids resulting from mating the $F_1$ hybrids to the parent strain L were intermediate in degree of susceptibility between the $F_1$ and the strain L animals. Of the group killed at 15 months, 15 percent had tumors, and of those killed at 18 months 22 percent had tumors.

It is obvious that when the tumor incidence was correlated with age, the results were not what one would expect if the character were inherited as a single dominant or recessive factor (fig. 1). The results, however, do suggest multiple-factor inheritance tending to fall in line with those obtained for induced tumors. In both cases the $F_1$ hybrids were intermediate to the two parent strains although approaching the degree of susceptibility in the parent strain A. Furthermore, with both the induced and spontaneous tumors, the L-backcross hybrids were intermediate between the $F_1$ and the parent strain L.

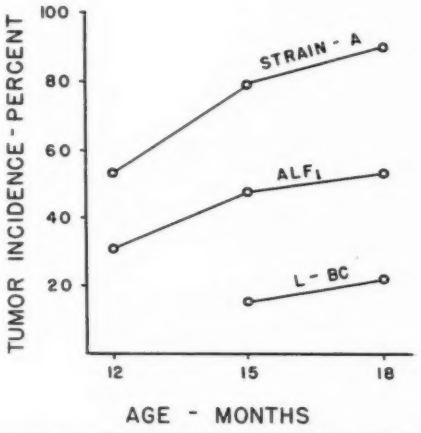


FIGURE 1.—Tumor incidence of strain A and of $ALF_1$ and L-backcross hybrids at definite age levels.

In the work with induced tumors it was found that an analysis could be made by measuring susceptibility on the basis of the number of nodules per individual. When susceptibility was measured in this way, the picture presented was typical of that of quantitative inheritance on a multiple-factor basis and thus confirmed the results obtained by correlating tumor incidence with the time following the injection.

It would be difficult with spontaneous tumors to make such an analysis measuring susceptibility on the basis of number of tumor nodules. In this experiment there were fewer animals with tumors than there would have been had the tumors been induced. Furthermore, most of the mice with tumors had but one nodule, the greatest number observed in any one individual being four. There was a tendency for the $F_1$ hybrids to develop fewer spontaneous nodules per individual than did the strain A mice, just as the $F_1$ hybrids developed fewer induced nodules than did the strain A animals. Also, in the L-backcross hybrids the number of spontaneous nodules per individual tended to be less than that of the $F_1$ hybrids, and this was likewise comparable with the results obtained with induced tumors.

The data of the experiment are not so extensive as would be desirable for a complete analysis of the inheritance of susceptibility to spontaneous pulmonary tumors. They do, however, show that neither a single dominant nor a single recessive factor can be involved, primarily since the $F_1$ hybrids are intermediate to the parent strains. However, the possibility of a single factor with incomplete dominance is not ruled out. Yet by the manner in which the data parallel those for induced pulmonary tumors, it seems very probable that the inheritance of spontaneous tumors, like that of induced tumors, is controlled by multiple factors.

It is well to point out that multiple-factor inheritance offers an explanation for the variation in susceptibility to spontaneous pulmonary tumors that has been observed between the different inbred strains (*5–7*). If a single factor were involved, one would expect the inbred strains to fall into two classes, one susceptible and the other resistant. Such, however, is not the case. While some strains have a very high tumor incidence and others a very low incidence, a number of the inbred strains show incidences intermediate to the two extremes. Strain A is well known as a high tumor strain, as various workers have published tumor incidences ranging from 70 to 90 percent. Strains C57 Black and L, on the other hand, have extremely low incidences, below 1 percent. Many other strains are neither strictly high nor low. From Andervont's data one finds that strain C mice have an incidence of from 20 to 30 percent; strains I and Y, from 10 to 20 percent; and strain C3H, from 5 to 10 percent. Lynch reported an incidence of 4.4 percent for her strain 1194, and of 43.9 percent for her strain Swiss–8. While strain 1194 is comparatively low, the Swiss–8 strain could hardly be considered high when compared with strain A.

It is well recognized that environmental factors influence the development of spontaneous pulmonary tumors. However, it does not seem probable that these intermediate tumor incidences appear because of variations in environmental factors alone. Many of the strains have been reared in the same laboratory and under the same conditions. The more probable explanation is that these variations in percentages are due in part at least to variations in gene complexes between the respective inbred strains. The situation might be compared with the inheritance of otocephaly and polydactyly in the guinea pig (*8, 9*). These are characters which are inherited as multiple-factor traits and for which different strains (or families) show different incidence percentages.

## SUMMARY

The data presented here, in which tumor incidence was correlated with age, along with the manner in which they tend to parallel the results published for induced pulmonary tumors, suggest that susceptibility to spontaneous pulmonary tumors in mice, like susceptibility to induced pulmonary tumors, is inherited on a multiple-factor basis.

It is pointed out that multiple factor inheritance offers an explanation for the variation in spontaneous pulmonary tumor incidence between the different inbred strains of mice.

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# Limits of Accuracy in Experimental Carcinogenesis as Exemplified by Tumor Induction With Ultraviolet Radiation

By HAROLD F. BLUM, *senior pharmacologist*, HUGH G. GRADY, *acting assistant surgeon*, and JOHN S. KIRBY-SMITH, *assistant physicist*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

The difference in time of development of tumors among individual animals subjected to the same treatment is the principal factor limiting the accuracy of quantitative studies of carcinogenesis. In the present paper, this factor is evaluated for the induction of tumors by ultraviolet radiation, where the variation in individual induction time within groups of animals is found to be very wide, necessitating the use of large numbers if significant group comparisons are to be made. While the limiting values found apply strictly to these experiments only, it seems probable that they are paralleled in carcinogenesis by other agents. At least the same or similar methods of analysis may be applicable elsewhere, and, therefore, they are presented in some detail. The method and accuracy of dosage with ultraviolet radiation and criteria for the determination of individual induction time have been discussed in previous papers (*1*, *2*).

## DISTRIBUTION OF INDUCTION TIME AMONG INDIVIDUAL MICE

The distribution of the time required for induction of tumors by ultraviolet radiation among the individuals of similarly treated, genetically identical groups of mice (strain A) is illustrated in figures 1 and 2. By plotting the logarithm of the induction time against the percent of animals with tumor (fig. 1), S-shaped curves are obtained which appear symmetrical. If percentages are translated into probability units (probits (*3*)), straight lines result as shown in figure 2. Both types of curve represent integrations of normal distributions.

The normal distribution of the logarithm of an attribute is common in biologic systems and has been found by Bryan and Shimkin (*4*) to apply to chemical carcinogens. The occurrence of this type of distribution has not been clearly explained and will not be discussed at this time. (But see Blum, Grady, and Kirby-Smith (*5*).)

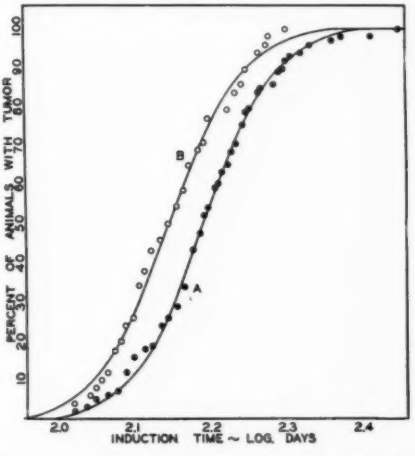


FIGURE 1.—Distribution of induction times for groups of mice subjected to ultraviolet radiation. Curve A represents 100 identically treated mice (groups AR, AS, AT, and AU, table 1); curve B, 51 identically treated mice (groups BE and BF, table 1).

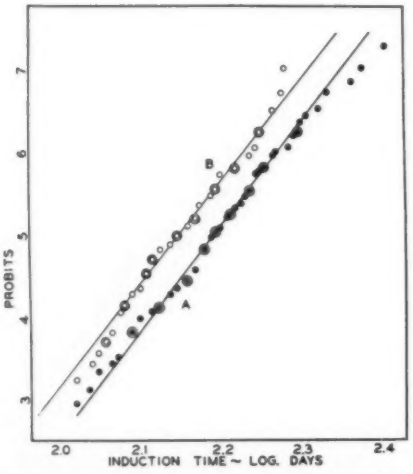


FIGURE 2.—Data of figure 1 plotted in probability units (*3*). The positions of the regression lines were calculated from the ringed points.

Figures 1 and 2 indicate normal distribution for two experimental groups, which received different treatment. That this distribution is characteristic of all the material studied is shown by the histogram in figure 3. This is based on experiments with 27 groups which have reached completion, i. e., all the surviving animals in these groups have developed tumors. The

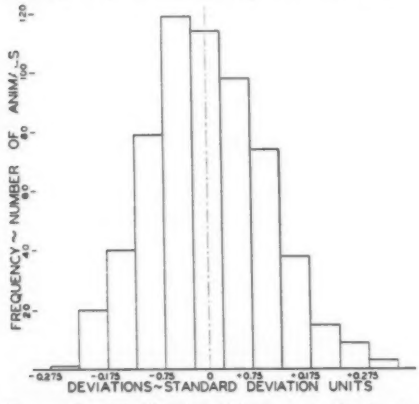


FIGURE 3.—Distribution of frequency of induction time among 613 mice, calculated from group means.

original number in each group was 26, making a total of 702, of which 613 survived until tumors appeared. In the figure, deviations from group means of the logarithm of induction time are plotted in standard deviation units against frequency represented by number of animals. This method of plotting permits the comparison of groups that received widely different dosages of radiation, for which the mean induction times were very different.

The distribution represented by the histogram is nearly normal, with a slight skew indicating a preponderance of individual induction times shorter than the mean. Assuming that the distribution is essentially normal, this skewing may be accounted for in a number of ways. The longer an experiment continues, the greater the likelihood that animals will die before they develop tumors. Thus, mortality, although not caused by the tumor-inducing agent itself, would tend to reduce the number of late tumors, this being enhanced by the logarithmic nature of the distribution, since in the histogram the positive portion not only includes an older group of animals but represents a greater elapsed time than the negative portion. In experiments with higher dosages, in which the induction times are shortest, mortality has little or no effect but may play a significant role in the longer, lower dosage experiments. Old mice develop tumors at somewhat slower rates than do young animals, and hence the last animals of a series, particularly in the case of low dosages, might require an inordinately long time for tumor induction. This would tend to skew the distribution in the manner indicated by the histogram. The tumor population is not entirely homogeneous (*2*), and this may also influence the distribution to a limited extent.

Such effects seem to be of minor importance on the whole, and normal distribution of the logarithm of the induction time may be assumed for purposes of analysis.

### The Slope of the Regression Line

By plotting probits for percents the sigmoid curves shown in figure 1 are transformed into the straight lines shown in figure 2. This method of plotting, developed by Bliss (*3*), has been applied to data for the induction of tumors by chemical carcinogens by Bryan and Shimkin (*4*).

Certain difficulties arise in applying this analysis to the present material. The method was designed for the treatment of points, each of which is obtained from a separate group of animals. By weighting each point according to the number of animals used in its determination and according to its position on the probit scale, the regression line which best represents the points may be determined. A method for accomplishing this has been worked out by Bliss, to whose paper reference must be made for details of the procedure (*3*). In the present case, the points represent one or a few animals within a single group. If the total number in the group were large enough and each point based on a sufficient number of animals, these data might be safely treated in the manner of Bliss. For example, if several groups of mice were similarly dosed but the groups killed off at different times and the number of tumors determined, one experimental point would be obtained for each group, and these points might legitimately be so treated. However, such an experiment involves difficulties, since it cannot be carried out efficiently without previous information making it possible to estimate appropriate times for killing the animals.

A cumulative error enters when our data are plotted in this fashion due to the fact that each point represents an integration of all those preceding it. This may give rise to apparent "rhythms," as in the curves in figures 1 and 2, and when small numbers are used may cause considerable deformation. The error manifests itself particularly at the extremes, but it has relatively little effect at the midpoint.

To avoid the influence of the extremes in calculating the regression line, certain points were selected according to an arbitrary scheme and the remainder disregarded. When considering groups of 26 animals or less, the values nearest 20, 40, and 80 percent were used, and where larger groups of animals were identically treated each 10-percent point was used. (See figure 2.) In a few instances it seemed best to select an adjacent point where that chosen arbitrarily was obviously less representative of the data. In calculating the position of the regression line, these points were weighted according to Bliss' method (*3*). The number of surviving animals was used in determining the weighting value, as in calculating the percent of animals with tumor (*1*).

Table 1 gives the slopes of the regression lines for experiments involving a variety of dosages. A few groups have been included which have not reached completion but for which the position of the regression line was estimated from those points obtained. The slope of the regression line varies but obviously is not correlated with either weekly dose, interval between exposures, or intensity.

Under ideal conditions the slope of the regression line is the reciprocal of the standard deviation. Hence, it is possible to compute the slopes from the standard deviations (table 1). Although slope values obtained in this way are free from cumulative error, they are greatly influenced by the extreme points and show a some-

TABLE 1.—*Assembled data for all experimental groups of mice under various conditions*

| Group | Exposures per week | Weekly dose, ergs per square centimeter $\times 10^7$ | Intensity ergs per square centimeter per second $\times 10^4$ | Slope of regression line | Standard deviation | $t_{50}$ | $t'_{50}$ | Age at $t_{50}$ | Animals surviving or with tumors | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | At first tumor | At $t_{50}$ | At last tumor |
| | *Number* | | | | *Log units* | *Days* | *Days* | *Months* | *Number* | *Number* | *Number* |
| BE | 7 | 13.8 | 4.3 | 15.0 | 0.074 | 142 | 138 | 7–8 | 26 | 24 | 24 |
| BF | 7 | 13.8 | 4.3 | 11.1 | .073 | 141 | 139 | 7–8 | 25 | 24 | 23 |
| BG | 7 | 6.8 | 4.3 | 14.9 | .080 | 165 | 161 | 8–9 | 25 | 24 | 24 |
| BH | 7 | 6.8 | 4.3 | 8.7 | .100 | 175 | 171 | 8–9 | 26 | 24 | 24 |
| BI | 7 | 3.7 | 4.3 | 9.1 | | | [1] 219 | 9–10 | 25 | 20 | |
| BJ | 7 | 3.7 | 4.3 | 16.0 | | | [1] 227 | 9–10 | 26 | 21 | |
| A | 5 | 43.0 | 4.3 | | | | [2] 166 | 8–9 | 23 | 18 | 16 |
| B | 5 | 16.5 | 1.6 | 13.2 | .066 | 160 | 154 | 7–8 | 25 | 25 | 25 |
| F | 5 | 16.5 | 4.3 | 11.6 | .090 | 146 | 142 | 7–8 | 24 | 24 | 24 |
| AR | 5 | 13.0 | 4.3 | 13.9 | .088 | 162 | 156 | 7–8 | 25 | 25 | 25 |
| AS | 5 | 13.0 | 4.3 | 14.6 | .086 | 157 | 150 | 7–8 | 25 | 25 | 25 |
| AT | 5 | 13.0 | 4.3 | 10.8 | .081 | 157 | 155 | 7–8 | 24 | 24 | 24 |
| AU | 5 | 13.0 | 4.3 | 11.2 | .082 | 158 | 155 | 7–8 | 26 | 25 | 24 |
| C | 5 | 9.9 | 1.0 | 13.7 | .058 | 151 | 145 | 7–8 | 26 | 26 | 26 |
| G | 5 | 9.9 | 4.3 | 13.6 | .081 | 154 | 148 | 7–8 | 26 | 24 | 24 |
| X | 5 | 9.9 | 4.3 | 14.0 | .086 | 204 | 198 | 17–18 | 20 | 20 | 18 |
| AA | 5 | 7.9 | .63 | 12.1 | .080 | 163 | 161 | 7–8 | 24 | 23 | 23 |
| AE | 5 | 7.9 | 1.0 | 11.1 | .087 | 192 | 186 | 8–9 | 24 | 23 | 23 |
| AI | 5 | 7.9 | 2.0 | 13.9 | .077 | 164 | 164 | 7–8 | 25 | 25 | 25 |
| AB | 5 | 5.3 | .42 | 14.0 | .086 | 213 | 213 | 9–10 | 25 | 25 | 23 |
| AF | 5 | 5.3 | 1.0 | 14.3 | .075 | 211 | 211 | 9–10 | 26 | 24 | 20 |
| AJ | 5 | 5.3 | 2.0 | 13.0 | .087 | 215 | 206 | 9–10 | 26 | 25 | 25 |
| D | 5 | 3.8 | .37 | 7.0 | .134 | 328 | 347 | 13–14 | 26 | 24 | 15 |
| H | 5 | 3.8 | 4.3 | 13.0 | .100 | 283 | 287 | 11–12 | 26 | 24 | 22 |
| AC | 5 | 3.6 | .29 | 14.1 | .070 | 336 | 336 | 13–14 | 22 | 21 | 19 |
| AG | 5 | 3.6 | 1.0 | 9.6 | .089 | 281 | 277 | 11–12 | 25 | 23 | 22 |
| AK | 5 | 3.6 | 2.0 | 11.3 | .080 | 254 | 250 | 10–11 | 22 | 21 | 20 |
| AD | 5 | 1.6 | .13 | | | | | | | | |
| AH | 5 | 1.6 | 1.0 | | | | | | | | |
| AL | 5 | 1.6 | 2.0 | | | | [2] 428 | | 22 | | |
| J | 1 | 8.6 | 4.3 | 7.7 | .107 | 244 | 240 | 10–11 | 25 | 25 | 23 |
| AN | 1 | 7.5 | 4.3 | 9.0 | .102 | 270 | 266 | 11–12 | 26 | 20 | 20 |
| AO | 1 | 4.3 | 4.3 | 16.7 | .062 | 294 | 290 | 12–13 | 25 | 25 | 23 |
| AP | 1 | 2.2 | 4.3 | | | | [2] 418 | | 20 | | |

[1] Obtained by interpolation.

[2] Obtained by extrapolation.

what wider variation than those obtained by the foregoing method; but like those values they indicate no systematic variation with experimental conditions. The standard deviation and values obtained from it should be little influenced by deaths among the animals, since these apparently do not result from the action of the radiation. On the other hand, the slope and position of the regression line are considerably affected by mortality. To permit an estimate of this factor, the numbers of animals surviving or with tumor in each group at various times are presented in table 1.

## COMPARISON BETWEEN GROUPS

Since approximately normal distribution is consistently followed, points representing the same percent, or corresponding probit value on regression lines, indicate that individual animals have the same susceptibility. The fact that the logarithm of the induction time, rather than the induction time itself, is distributed normally does not affect this argument. Hence, the time required to induce tumors in a given percent of the animals of a group may be used as a measure of tumor induction for purposes of comparison among groups subjected to different dosages or differently treated in other ways.

If the slope of the regression line is constant, any corresponding percent points may be used for comparison. Actually, however, there is a certain amount of variation of the slope; and since the

induction time for 50-percent tumor incidence is the most accurately established, it is the best to use. For practical purposes this may be obtained either from the mean of the logarithms of the induction times, or from the regression line by interpolation. The former will be designated $t_{50}$, the latter $t'_{50}$. Both values are presented in table 1; $t_{50}$ should be the more accurate, but $t'_{50}$ is easier to obtain and may be more useful. Actually, the two values differ very little in most of our experiments. (See below.) With correspondingly less accuracy it is possible to obtain $t'_{50}$ by extrapolation from earlier points when less than half the animals have developed tumors.

## ACCURACY OF COMPARISON

When two experimental groups are to be compared, the significance of the difference between the two values of $t_{50}$ can be estimated from the standard deviations and numbers of animals in the two groups by ordinary statistical procedure (*6, pp. 128–129*). In designing experiments to study the effects of a given factor, say dosage or diet, the question arises: How many animals must be used in each group to assure a given degree of accuracy?

Table 2 has been devised to answer this question by indicating the accuracy to be expected when various numbers are used. It shows, for given numbers of animals in each group, the percent by which one value of $t_{50}$ must exceed another if the two are to be considered significantly different, i. e., such a difference will not occur more frequently than once in 20 times due to chance alone. In calculating these values, the average of all values of the standard deviation in table 1 (0.084 log units) has been used in the equation for the significance of the difference between two means (*6, pp. 128–129*). It is possible to present the value by which one mean must exceed another in percent because the logarithm of the induction time is distributed normally, and hence the deviation between the means calculated from this equation is a logarithm, which represents a definite fraction. To give a more concrete idea of the extent by which means may differ without the difference being significant one value of $t_{50}$ is set at 200 days, and the number of days by which the second value must exceed it in order to be significant is estimated in the third column of table 2. If the data of this table are compared with those for the dosage relationships presented in table 1 (see (*5*)), it is obvious that unless rather large numbers of animals are used, the allowable difference is such a large fraction of the working range that little information can be expected from a single experiment.

The estimates of allowable deviation presented in table 2 must be regarded as minimum values. At best they are only valid for $t_{50}$, i. e., the antilogarithm of the mean of the logarithms of the induction times for completed experiments. Cumulative error, and mortality (p. 86) do not affect this value. On the other hand, $t'_{50}$, which is obtained by interpolation, is subject to additional uncertainty arising from these sources. To estimate the extent of this uncertainty the percent deviations of $t'_{50}$ from $t_{50}$ were determined from the data in table 1, and the mean

TABLE 2.—*Number of animals required for statistical significance*

| Animals in each group | Percent by which one value of $t_{50}$ must exceed another to be significant | Minimum difference necessary for significance when lowest value of $t_{50}$ is 200 days |
|---|---|---|
| *Number* | | *Days* |
| 10 | 20 | 40 |
| 15 | 16 | 32 |
| 20 | 13 | 26 |
| 25 | 11.5 | 23 |
| 50 | 8 | 16 |
| 100 | 5.5 | 11 |

and standard deviations were calculated; $t'_{50}$ is less than $t_{50}$ except in a few cases, and the difference is expressed by $t'_{50} = t_{50} - 1.7$ percent $\pm 2.3$ percent. Thus the two values are not strictly comparable; and if values of $t'_{50}$ are used, it would seem wise to extend the allowable difference by about 5 percent, although some of this difference may represent variability in $t_{50}$ itself rather than in $t'_{50}$. These estimates apply only to experiments in which about 25 animals are used in each group; with larger numbers of animals the cumulative and mortality errors should be less, and with smaller numbers they should be much greater.

Another manner of comparison, and one not infrequently used in certain types of biologic experiment, is to compare for two groups the times at which 50-percent incidence is reached or surpassed. Designating this value as $t''_{50}$, our data show that $t''_{50} = t_{50} - 0.5$ percent $\pm 4.0$ percent. This indicates that this value is considerably less satisfactory than $t'_{50}$. Use of $t''_{50}$ enjoys the advantage, however, that the experiment may be terminated after 50-percent incidence is reached, whereas $t'_{50}$ requires that a greater percent incidence be attained, and $t_{50}$ requires that all animals must develop tumors. Thus, while time may be saved by curtailing the length of the experiment, this is done at the sacrifice of accuracy, and a greater difference between significant values must be accepted, or the number of animals must be substantially increased.

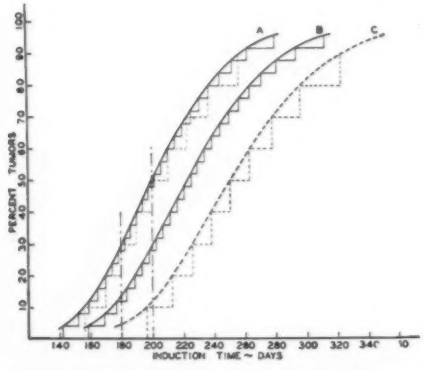


FIGURE 4.—Curves A and B indicate the limit of significance for 2 groups of 25 animals each when $t_{50}$ is 200 days for 1 group (A). The steps represent individual animals. Curves A and C indicate the limit of significance for 2 groups of 10 animals each. The dotted steps represent individual animals.

It is rather common practice in cancer research to compare two groups of animals by examining them at some arbitrary time and comparing the percentages of animals found to have tumors in each group. Certain of the errors involved in this method are illustrated in figure 4. Curve A represents the distribution of induction time to be expected when $t_{50}$ is 200 days, and curve B when $t_{50}$ is 223 days. These 2 curves correspond to the limits which may be allowed between values of $t_{50}$ for 2 groups of 25 animals without recognizing them as significantly different. Steps of 4-percent incidence have been drawn, each of which represents 1 animal in a group of 25. If the animals in such groups were examined at 180 days, for example, the group represented by curve A would show 28 percent tumors, that represented by curve B, 12 percent; if examined at 200 days, the values would be 48 and 28 percent, respectively. These differences seem rather large but are without significance under the conditions described. Actually, considerably greater differences would not be significant because the curves drawn are assumed to be equally stable at all percentages, whereas much greater variations may occur at points distant from 50-percent incidence. Because of the logarithmic distribution of induction time, these differences might be even greater for experiments in which the tumors appear more slowly, i. e., when

$t_{50}$ is greater. Comparison of curves A and C illustrates the condition when groups of 10 animals instead of 25 are used. Obviously, when such small numbers are used, the experiments are difficult to interpret. Such a method of comparison is unsatisfactory unless very large numbers of animals are used and great care is taken in selecting the time for comparison.

Our data indicate that experiments need not be carried out concurrently. Needless to say, however, if they are not so performed the genetic purity of the mice, control of dosage, and constancy of diet must all be carefully standardized. There may be a certain advantage in using smaller groups of animals since it is possible to obtain them of more nearly the same age. It seems, however, that groups smaller than 25 are not to be recommended, and probably at least 2 groups of this number must be used to obtain satisfactory results.

## SUMMARY

The results of a considerable number of experiments on the induction of tumors by ultraviolet radiation are examined statistically to determine the accuracy to be expected in such studies.

The logarithm of the induction time is normally distributed among individual male strain A albino mice. The distribution does not vary systematically with dosage of radiation, interval between exposures, intensity of the radiation, or age.

The accuracy to be expected when experimental groups are compared is considered, and different methods of comparison are evaluated. The variation among individuals is very wide, making it necessary to use large numbers of animals and to give particular attention to methods of comparison if a reasonable degree of accuracy is to be achieved.

Strictly, these findings apply only to induction of tumors by ultraviolet radiation, but it is probable that the wide variation found here is not unique in the field of cancer. If comparable latitude is found elsewhere, certain types of comparison in common use may prove unreliable.

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# Relationships Between Dosage and Rate of Tumor Induction by Ultraviolet Radiation

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The preceding paper (*1*) deals with statistical aspects of the data obtained from a series of experiments in which tumors were induced with various dosages of ultraviolet radiation and provides a basis for estimating the accuracy of the results. The present paper deals with relationships between dosage and rate of tumor induction demonstrated by these experiments.

## RESULTS

As explained in the preceding paper, the time required to induce tumors in 50 percent of the animals of a group, if properly measured, is the best means of comparing the rate of tumor induction for different groups. This time to 50-percent tumor incidence will be designated as $t_{50}$,[1] the individual induction time as $t$.

In figure 1, $t_{50}$ is plotted against the weekly dose; the relationships displayed will be discussed separately with regard to various dosage factors.

### EFFECT OF DOSE

The most complete data are those for 5 exposures per week (each day except Saturday and Sunday). These are represented by the solid disks in the figure. Each point is based on between 50 and 100 animals, with the exception of those at the extremes of the curve which are not highly reliable, each being based on less than 25 animals and obtained by extrapolation. These extreme values represent the limits of dosage within which it is possible to work; since the higher dosage eventually causes death and when the lower dosage is employed, tumors are so late in appearing that the average life span of the animals may be reached before a significant number develop tumors. Moreover, the curve is so steep at such low doses that small errors in reproducing the dosage become an important source of error.

The most striking feature of the dose-$t_{50}$ relationship indicated by the curve drawn through the five-exposures-per-week points, is that above a certain dose (approximately $10^8$ ergs per square centimeter per week) a minimum time is re-

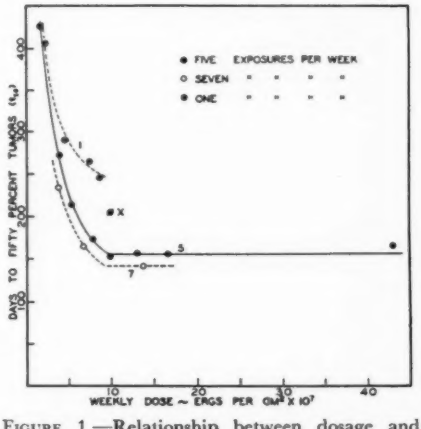


FIGURE 1.—Relationship between dosage and time to 50-percent tumor incidence.

[1] $t_{50}$ *is used where available,* $t'_{50}$ *in a few instances. See the preceding paper* (1) *for explanation of these symbols. While* $t_{50}$ *is not the average of the induction times, it may be so regarded if it helps to clarify the present analysis. Quantitatively, it is the antilogarithm of the mean of the logarithms of the individual induction times.*

quired for tumor induction which cannot be significantly decreased by increasing the dosage. Below this dose, the time increases with decreasing dosage, the curve seeming to approach an asymptote which suggests a minimum dosage necessary for tumor induction.

### Effect of Interval Between Exposures

Although increasing the weekly dosage does not reduce $t_{50}$ below a certain minimum value so long as the schedule of 5 exposures per week is maintained, increasing the schedule to 7 exposures per week accomplishes such a decrease in induction time. Approximately 50 animals were used in establishing each of the 3 points shown in figure 1 for this schedule of dosage, and the differences from the 5-exposures data are significant. They show that the same weekly dose is always more effective when administered in 7 exposures than when administered in 5. While the points are too few to determine the exact shape of the line relating dose to $t_{50}$, they indicate that a minimum induction time obtains for 7 weekly exposures as well as for 5, as is suggested by the dotted line drawn through these points in the figure.

In corresponding fashion, if the weekly dose is administered in a single exposure, $t_{50}$ is longer than if the same dose is divided over 5 or 7 days, as is indicated in the figure. Only 20 to 25 animals were used in determining each of these points, but the differences from the 5-exposures-per-week data are highly significant in the case of the 3 highest dosages. The lowest dosage point was obtained by extrapolation and is not accurately located. The dotted line drawn through the points obtained for this schedule of exposure suggests a minimum induction time in this case as well, which is greater than when the exposures are more frequent.

### Effect of Age

The experiments were carried out on young mice between 2 and 3 months of age at the beginning of the experiment, with one exception (X, figure 1, and group X, table 1 (*1*)) in which the animals were approximately 10 months old. For these older mice $t_{50}$ was much greater than for young animals receiving the same dosage. The difference is highly significant, and there can be little doubt that tumors are induced more slowly in mice of this age than in young mice.

### Effect of Intensity

Certain of our experiments were designed to examine the effect of intensity of radiation by comparing series which received equal dosage but applied at different intensities. (See Blum, Kirby-Smith, and Grady (*2*) for the method.) It was feasible to vary the intensity widely only when relatively low doses were used, since to obtain high dosage at low intensity it would have been necessary to prolong the periods of exposure to such an extent that the availability of the apparatus for other experiments would have been too greatly restricted. Thus, a completely satisfactory answer to this important question has not been obtained. (See preceding paper (*1*).) However, between the intensities $4.3 \times 10^4$ and $4.2 \times 10^3$ ergs per square centimeter per second—a tenfold range—no significant differences were found. Two groups which were exposed to intensities lower than the latter value showed significantly higher $t_{50}$ values than groups which received the same dose at higher intensities.[2] The dose to which these groups were exposed corresponds to a steep region of the curve at which reproduction of dosages is more critical

[2] *See table 1 of the preceding paper. These low intensity groups (D and AC) were not included in the data from which figure 1 was prepared.*

than at higher values. The statistical treatment (*1*) assumes that dosage is measured accurately, and at higher dosages this assumption is justified. At these low dosages, however, it is possible that error in dosage measurement may enter and this is not taken into account by the statistical treatment. Hence, the estimate of significance in this particular case may be questioned.

It is important, for practical purposes, to know that the intensity may be varied within rather wide limits without affecting $t_{50}$ to a significant extent but that it is unwise to permit too great fluctuations. For example, such fluctuations as have occurred in the course of our experiments due to uncontrolled changes in the mercury arc are obviously without significant effect on the results; but had a less stable source of radiation been used, the reliability of the data might be considerably less (*2*).

## FACTORS INFLUENCING THE DOSE-RATE RELATIONSHIP

No equation based on a simple mechanism is found to describe the relationship between dose and rate of tumor induction. This is not surprising when one considers the numerous factors that may affect both dosage and rate. It is important to discuss some of these factors.

### Screening Effect of the Epidermis

The effective dose is determined by a factor that controls the amount of radiation reaching the tissues in which the tumors are induced. Of the radiation incident on the mice's ears, a certain large fraction is absorbed by the dead corneum which thus acts as a semi-opaque screen to protect the living cells of the underlying tissues.

The logarithmic distribution of the induction time pointed out in the preceding paper (*1*) could be explained by this screening action. If the effective radiation of a given wavelength, $I$, is that which passes through the nonparticipating layer, it is related to the incident radiation $I_o$ according to the Bouger-Lambert law

$$I = I_0 \times 10^{-kl} \tag{1}$$

where $l$ is the thickness of the nonparticipating layer, and $k$ its attenuation[3] coefficient.

Assuming that the incident radiation is constant for all the animals of the group (*1*),

$$I \propto 10^{-kl} \tag{2}$$

or

$$-\log I \propto kl \tag{3}$$

If the tumor induction time is inversely proportional to the effective radiation, which is the simplest assumption that can be made,

$$t \propto \frac{1}{I} \tag{4}$$

or, combining with (4)

$$\log t \propto kl \tag{5}$$

This treatment, which applies to any given wavelength, may be extended to include all the wavelengths to which the animals are exposed, by properly adjusting the value of $k$.

Hence, if $kl$ is distributed normally among the group, the logarithm of the tumor induction time must also be normally distributed, as is shown by our data to be true experimentally. It seems probable that $k$ is very nearly constant for a given wavelength and that differences in transmission must depend principally upon the thickness, which would probably be distributed normally. Thus, the hypothesis that absorption of radiation by the outer nonparticipating layers of the skin conditions the observed normal distribution of the logarithm of the induction time is plausible, since a large fraction

[3] *The attenuation coefficient recognizes both absorption and scattering and may be used instead of the absorption coefficient when dealing with such a system.*

of the incident radiation of wavelengths that induce tumors is absorbed in the epidermis (*3*).

However, our measurements do not suggest enough variation in epidermal transmission from mouse to mouse to account alone for the wide variation in induction time, and it is possible that the filtering action of the epidermis is negligible in its effect upon this distribution.

If this factor can be disregarded, the distribution must be determined chiefly by inherent biologic differences. This is not improbable since this type of distribution is frequently met with in the dose-response relationships of drug action (*4–6*) and elsewhere in biologic systems although it is seldom as clear-cut as in the present case. It is particularly significant that this type of distribution is found in the dose-response relationships of carcinogenic chemicals (*7*).

Whether or not the filtering action of the epidermis plays an important role in determining the distribution of induction time among the individuals of a group, it may have a role in determining the dose-$t_{50}$ relationship which must not be overlooked. Ultraviolet radiation brings about thickening of the epidermis and hence reduces its transmission for this radiation. The layer of viable cells of the epidermis increases to as much as three or four times its thickness in instances where high doses are administered, and the corneum, which is chiefly responsible for the reduction in transmission, is likewise thickened. Histologic examination of the skin shows that the degree of thickening of the epidermis approximately parallels the dosage but does not permit quantitative evaluation of this relationship.

Obviously, the fraction of such radiation reaching the subepidermal tissues may be expected to vary with the degree of thickening and hence with the dose, but without further data it is impossible to estimate the magnitude of the effect on the dose-$t_{50}$ relationship.

## Components of the Induction Time

The nature of the induction time and modifying factors must next be considered. What we have called the individual induction time, $t$, is the total period between the first exposure of the animal to radiation and the time the tumor reaches a given size. (See Grady, Blum, and Kirby-Smith (*8*).) This may be logically divided into two portions for purposes of analysis: $t_i$, the time required to initiate the tumor, and $t_g$, the additional time required for growth to the required size.
Thus

$$t=t_i+t_g \qquad (6)$$

Obviously, there is no way to estimate $t_i$ and $t_g$ directly, but certain possibilities may be tested.

Let us assume that $t_i$ is a constant fraction of $t$, i. e.,

$$t=t_i\times k \qquad (7)$$

In this case $t_g$ must also be a definite fraction of $t$. The logarithmic form of (7) is

$$\log t=\log t_i+\log k \qquad (8)$$

and in this case, if the logarithm of $t$ is normally distributed, the logarithm of $t_i$ must also be normally distributed, for the addition of log $k$ does not affect this distribution. In the log $t$-probit graph (*1*, *fig. 2*), log $k$ will not influence the slope of the regression line but will only affect its position on the abscissa.

This relationship seems the most probable; and if it obtains, the criterion of tumor selection (*8*) is strictly valid—in fact, it would make no difference what tumor size is adopted as the end point in determining $t$, so long as the end point itself is uniform.

An alternate possibility is that $t_g$ is con-

stant and independent of $t_i$; in other words, after the tumor is initiated, it grows at an average rate which is not affected by the amount of radiation previously received or by further irradiation. In this case

$$\log t = \log (t_i + t_g) \qquad (9)$$

and if this were the case, normal distribution of log $t_i$ would not be reflected in normal distribution of log $t$, so that the log $t$-probit relationship would deviate from a straight line to a degree determined by the magnitude of $t_g$.

By assigning values to $t_g$, it is possible to show by graphical analysis that neither deviations from linearity nor apparent changes in slope of the regression line would be detectable unless $t_g$ is given a value of about 60 days, so that no conclusive answer can be obtained in this way. However, a systematic change in slope would be expected as $t_{50}$ varies, and this is not evident in our data.

Thus, while the alternate hypothesis cannot be definitely ruled out, the concept that the growth and induction periods are both proportional to the total measured induction time and hence that the latter is a measure of the tumor induction process, seems the most reasonable.

### Hyperplasia

As previously pointed out, hyperplasia of the epidermis and accompanying thickening of the corneum may affect induction time by altering the amount of radiant energy reaching the living cells. Just what effect the hyperplasia itself has on the process of tumor induction is a question of some importance, but one to which our experiments provide no definite answer. At higher doses the tumors are always associated with a considerable degree of hyperplasia of both the epidermis and the deeper tissues, which is present almost from the beginning of the exposures. On the other hand, at lower doses only very slight hyperplasia may be observed, even up to the time the tumor appears. In fact, some of the tumors appear as localized lesions on the ear the remainder of which appears grossly normal and exhibits little microscopic change. Thus, it is difficult to say that hyperplasia is an essential factor for tumor formation, or even that the two processes are directly related. On the other hand, hyperplasia may well be a modifying factor in tumor induction. The mere fact that this process provides cells upon which the carcinogenic agent may act should not be overlooked in formulating any hypothesis regarding the mechanism of tumor induction or in interpreting quantitative relationships.

### Tissue Destruction

The fact that ultraviolet radiation is a lethal agent, which may actually accomplish its carcinogenic effects by partial damage to the cell, must not be overlooked. At high dosage, a considerable number of cells are destroyed and actual disappearance of parts of the ear may result from such damage followed by necrosis. Such destruction must affect the induction time, since it decreases the amount of tissue in which tumors may develop.

### Age

The age of the mice may affect the dose-$t_{50}$ relationship since older animals develop tumors more slowly (p. 92), and those which receive low dosage are older at $t_{50}$ than those receiving high dosages. It is possible that this is important only at relatively low dosages, but no accurate estimate of the effect on this relationship can be made from the available data.

The cause of this age effect is an interesting point for inquiry. It may result

because old mice are less susceptible to cancer than young mice, a rather generally suggested idea. Another factor, of undetermined relative importance, is epidermal transmission, which we have found to be considerably less in old mice than in young mice when neither have been subjected to ultraviolet radiation. Whether this difference is maintained after the animals have been irradiated we cannot say.

### Body Weight

Dosages above about $3 \times 10^7$ ergs per square centimeter per week cause a decrease in body weight of the mice, body weight and the logarithm of the dose being linearly related (*9*). This decrease in body weight can be accounted for by decreased food intake, and since Tannenbaum (*10*) finds that restriction of diet delays the appearance of certain types of tumors, this must be recognized as a factor that may modify the induction time.

## DISCUSSION

One aspect of the dose-$t_{50}$ relationship stands out, namely, the existence of a minimum induction time. That this does not represent an absolute minimum time required for tumor development is shown by the fact that the minimum shifts with the schedule of dosage, being less when the exposures are more frequent. A possible explanation is that carcinogenesis is limited by the saturation or exhaustion of some substance in the cell, which is renewed in the interval between exposures. The exhaustion or saturation of this hypothetical substance should set a limit for the amount of carcinogenic action achieved in a single exposure period; but if the exposures were more frequent, more carcinogenic action could result from the same dosage. Such a concept is in accord with the findings but is rendered somewhat unlikely by the fact that a minimum induction time is also encountered when chemical carcinogens are administered (*7*).

Another hypothesis may be offered, that tumor induction by ultraviolet radiation is associated with injurious changes in the cells which are reversible in the sense that the cell may recover from them. If the injurious changes are too severe, the cell is killed; if they do not exceed a certain degree, the cell recovers. Thus certain limits are imposed, i. e., only those cells which receive neither too much nor too little injury develop the characteristics of cancer cells. This would account for the minimum induction time, since increasing the dosage above a certain point would only result in the destruction of more tissue cells without increasing the proportion of potential cancer cells. Different intervals between exposures would permit different degrees of recovery, and hence the minimum induction time would shift with the schedule of dosage.

Undoubtedly there are other hypotheses which will also fit the data here presented, and the foregoing can only be regarded as suggestions that may aid in the design of further experiments to elucidate the mechanism of carcinogenesis.

Certain limitations to such studies are clearly shown by these data. If it is desired to study the action of a given factor, say diet, on tumor induction, dosages between $10^7$ and $0.4 \times 10^7$ ergs per square centimeter per week should be used. These dosages correspond to $t_{50}$ values of approximately 5 and 10 months, respectively, when given in five equal doses per week. Since the mice must be 2 to 3 months old at the beginning of the experiment, when the lower dosage is used, mortality may enter to an important extent. Moreover, errors in the reproduction of dosage conditions may be an important factor at this low dosage level, because the curve is so steep in this region

that small differences in dose have great influence on $t_{50}$.

Above $10^7$ ergs per square centimeter per week, increasing the dose does not increase the rate of tumor induction, and it is probable that other factors would have little effect on $t_{50}$ in this dosage region. However, the possibility that certain factors might alter the minimum $t_{50}$ value while others might not should be kept in mind in the design of experiments. On the whole, it would seem wise to explore both the dosage region above and below the minimum $t_{50}$ point in examining the effect of any modifying factor.

The limitations of the accuracy of the measurements of $t_{50}$, which must affect the usefulness of such a method of study, have been discussed in the preceding paper (*1*).

## SUMMARY

Relationships between dosage and tumor induction time have been explored.

The induction time is shorter when the same weekly dose is given in smaller, more frequent exposures.

For a given schedule of exposures there is a minimum induction time which is not reduced by increasing the weekly dose, but below a certain dosage the induction time increases rapidly with decrease in dose.

The quantitative relationships are not describable in terms of a simple mechanism. Numerous factors which may serve to mask such a relationship are discussed.

The induction time for old mice is longer than for young mice receiving the same dosage.

Within rather wide limits the induction time does not vary significantly with intensity of the radiation.

Certain hypotheses of the mechanism of carcinogenesis by ultraviolet radiation are considered. The experimental limitations of such studies are discussed.

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# Effect of Climatic Environment Upon the Genesis of Subcutaneous Tumors Induced by Methylcholanthrene and Upon the Growth of a Transplantable Sarcoma in C3H Mice[1,2]

By Edward W. Wallace, *associate professor of pharmacology*, Helene M. Wallace, *research associate*, and Clarence A. Mills, *professor of experimental medicine, College of Medicine, University of Cincinnati*

## INTRODUCTION

The experimental study of the effect of environmental temperature upon tumor genesis and growth divides itself into a study of (1) chemically induced tumors, (2) transplantable tumors, and (3) spontaneous tumors. The present paper deals with parts 1 and 2. Experiments upon the effect of environmental temperatures on spontaneous tumors of C3H mice are under way and will appear in a separate paper.

Previous work on the effect of climatic temperatures upon malignant growths has been for the most part on spontaneous cancers in man under uncontrolled conditions. Mountin and Dorn (*1*) and Gover and Collins (*2, 3*) have statistically analyzed cancer rates in the United States with respect to geographic variation and climate. Their data show a somewhat higher rate for the commoner types of cancer in the Northern States as compared with the Southern, with the exception of skin cancer which appears to be more prevalent in States with warmer climates.

Smith and Fay (*4*) have shown the effects of reduced temperatures directly applied to cancer in man and in embryonal cell growth. While their results appear to be striking, they are based upon inadequately controlled experimental material and deal with temperatures much lower and applied in a different manner than could be included under the term "climatic." Other workers attempting to repeat Smith and Fay's experiments by similarly subjecting experimental animals to very low temperatures have been unable to show an effect upon cancerous growth. These results were possibly due to too severe general chilling, chilling which was immediately lethal to a large percentage of animals, or to the necessarily intermittent and short duration of the exposures.

Banzer (*5*) recently reported a decreased incidence and rate of tumors in an animal colony he took on a cruise from Germany into the warmth of the Caribbean. However, the number of animals was small, and the experiment was poorly controlled both with respect to temperature and genetics.

Fuller, Brown, and Mills (*6*) have reported a study of the influence of climatic environment, constantly controlled, upon spontaneous tumors in dba mice. Unfortunately, because of a high noncancerous mortality as well as a relatively low inci-

[1] *This investigation was conducted in the Departments of Pharmacology and Internal Medicine, College of Medicine, University of Cincinnati, and was supported in part by a grant from the National Cancer Institute.*

[2] *Presented before the thirty-fourth annual meeting of the American Association for Cancer Research, Chicago, April 15–16, 1941.*

dence and variation in tumor type in this strain, the data are few, 4 tumors developing in the tropical temperature and 14 in the temperate. This experiment is now being repeated by the present authors on a large group of C3H mice, which are known to be susceptible to mammary carcinoma and in our hands more resistant to intercurrent infections in the hot and cold rooms.

## EXPERIMENTAL PROCEDURE

A litter of strain C3H mice was obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. From these mice a colony was built up by filial inbreeding, by using the methods employed at the Jackson Memorial Laboratory. All animals were bred, born, and reared up to the experimental period in a room kept constantly at 79° F. Humidity was approximately controlled between the limits of 20 to 40 percent saturation. By constant ventilation and filtration the air remained clean and odorless. The animals were kept in sanitary, vermin-proof, metal cages. The diet for all animals except during pregnancy, lactation, and before weaning consisted of Purina dog chow. During the lying-in period both mother and litter were fed a modified Sherman diet, adapted to mice and fortified especially with vitamins A, B, D, and E, and with iron, copper, and manganese. All food and water were given ad libitum and from overhead containers which kept contamination to a minimum. Under these conditions the animals remained in a vigorous, healthy state.

Andervont (*7*) recently reviewed the literature on tumor incidence in this strain of C3H mice. Contrary to the findings of some, in our hands virgin C3H females from the Jackson Memorial Laboratory showed a low tumor incidence, almost zero percent in 142 virgin females at 16 months when the animals were killed. However, both males and females were highly susceptible to the action of methylcholanthrene, and we were able to obtain tumors as readily and with the same latent period as found by others (*8*).

The hot and cold rooms in which the effects of environmental temperatures upon tumor genesis and growth were studied were controlled in a manner similar to that already described for the breeding and stock animals. The hot room was constantly held at 92° F. and at 50 percent relative humidity, and the cold room at 65°, without attention to the humidity, which was usually low.

These temperatures were selected as representing average tropical and temperate climates, respectively. They also represent about the extremes in which we have found that long-term experiments with mice may be carried out, since at a little over 92° F. fever becomes a complicating factor while below 65° the excessive chilling predisposes the animals to respiratory disease.

The rooms were illuminated artificially by an ordinary 60-watt Mazda light bulb which was kept on night and day. Thus, not only did the light factor and its effect upon activity of the animal remain constant, but also there occurred no radiation of ultraviolet light, a factor of significance in connection with the reported high incidence of skin cancer in the South and also the experimental initiation of skin cancers in animals by ultraviolet light (*9*).

In all of our experiments an acclimatization period of 1 month preceded the experimental procedures. During this time the general metabolism and heat loss mechanisms of the animals became adjusted from 79° F. to the tropical or temperate environments.

## CHEMICALLY INDUCED TUMORS

Healthy male C3H litter mates were set aside after weaning and reared at 79° F. until they were 3 months old, the age at which they become mature and the bulk of body growth is attained. In this way the possible effects of differential growth and sex factors in the hot and cold environments upon tumor genesis were avoided. At 3 months each litter was divided equally between hot and cold rooms. Thus the genetic constitution of the two groups was as nearly alike as possible, and the genetic factor in susceptibility to carcinogens was held constant. After the 1-month period of acclimatization, methylcholanthrene was injected subcutaneously, with antiseptic technique, into the right posterior lumbar area, and a weekly gross physical examination was made of the injection site thereafter.

Following some earlier experiments by Andervont (*10*), we used a dose of 2 mg. of 20-methylcholanthrene in 0.25 cc. of lard. The methylcholanthrene (m. p. 174°–176° C.) came from the chemical division of the Eastman Kodak Co., and the lard was the liquid portion (at 37°) of a commercial sample. Tumors may, of course, be obtained with smaller doses of methylcholanthrene (*11*) and by using other vehicles. However, since we started with this dose and since the results were positive and significant, we continued with the same dose in order to keep this factor constant and make our experiments comparable.

Table 1, experiment 1, summarizes the incidence distribution of effects produced upon some 26 pairs of C3H male mice. A four-plus system was used in recording appearance and progress of edema, alopecia, and induration. In the summarized table, however, the figures represent time of first appearance, i. e., one plus, of the symptoms in the number of animals shown. The most definite and significant finding, of course, was the appearance of a hard, palpable mass at the injection site. Confirmation of its malignancy came from its subsequent growth, which was

TABLE 1.—*Incidence distribution of effects produced in 4-months-old strain C3H male litter mates injected subcutaneously with 2.0 mg. of methylcholanthrene in 0.25 cc. of lard*

EXPERIMENT 1

| Environment | Effect | Number of mice | Incidence distribution after injection (in weeks) | | | | | | | | | | | | Negative | Positive | Average incidence |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | | | |
| | | | | | | | | | | | | | | | *Number* | *Number* | *Weeks* |
| Hot room | Edema | 26 | 3 | | | | | | | | | | | | 23 | 3 | 4.0 |
| | Alopecia | 26 | 5 | 5 | 1 | 3 | 4 | | | | | | | | 8 | 18 | 5.8 |
| | Induration | 26 | | | | 5 | 15 | 1 | | | | | | | 5 | 21 | 7.8 |
| | Mass | 26 | | | | | 2 | 12 | 7 | 3 | 1 | | | | 1 | 25 | 9.6 |
| Cold room | Edema | 27 | 1 | | | | | | | | | | | | 26 | 1 | 4.0 |
| | Alopecia | 27 | | 1 | | | 9 | | | | | | | | 17 | 10 | 7.7 |
| | Induration | 27 | | | | 1 | 9 | 3 | | 1 | 1 | 2 | | | 10 | 17 | 9.1 |
| | Mass | 27 | | | | | | 2 | 3 | 9 | 2 | 2 | 3 | 3 | 3 | 24 | 11.8 |

EXPERIMENT 2

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hot room | Edema | 27 | | | 23 | | 1 | | | | | | | | 3 | 24 | 6.1 |
| | Alopecia | 27 | | | 22 | | 1 | 1 | | | | | | | 3 | 24 | 6.2 |
| | Induration | 27 | | | | 12 | 9 | 3 | | | | | | | 3 | 24 | 7.6 |
| | Mass | [1] 22 | | | | | | 6 | 10 | 6 | | | | | | 22 | 10.0 |
| Cold room | Edema | 25 | | | 13 | 2 | 1 | 1 | 1 | | | | | | 7 | 18 | 6.6 |
| | Alopecia | 25 | | | 4 | | 1 | | | | | | | | 20 | 5 | 6.4 |
| | Induration | 25 | | | | 4 | 6 | 3 | 2 | 4 | 1 | | | | 5 | 20 | 8.9 |
| | Mass | 25 | | | | | | 1 | 1 | 10 | 7 | 3 | 1 | | 2 | 23 | 11.6 |

[1] 5 of the 27 animals escaped from an improperly closed cage, and data are therefore given for the 22 remaining.

carefully measured. The average latent period for tumor production was 9.6 weeks with 1 negative for the hot-room animals as compared with an average latent period of 11.8 weeks with 3 negatives for the cold-room animals.

In order to test the reliability of our results the experiment was repeated after 8 months on 25 additional litter-mate C3H male mice (table 1, experiment 2). The latent period in tumor production was longer, and there were more negatives in the cold room than in the hot. More than this, the results upon tumor genesis almost exactly duplicated the first experiment. The combined results of the two experiments were therefore examined statistically, and it was found that the mice in the hot room developed tumors in $9.78 \pm 0.17$ weeks, and those in the cold room in $11.70 \pm 0.22$ weeks, the observed difference of 1.92 weeks being 6 times the standard error of the difference and, therefore, highly significant.

A record of tumor growth was kept for each animal by measuring with a pair of calipers the major axes once a week. However, it soon became apparent, even grossly, that we were dealing with different types of subcutaneous tumors in the various animals. Some of the tumors were probably epithelial and ulcerated early. Others seemed to invade the muscle quickly and became irregular and difficult to measure. Histologic examination of subcutaneous methylcholanthrene-induced tumors (*12*) has shown that they arise from different cell types.

## TRANSPLANTABLE SARCOMA

To study in a quantitative way the effect of hot and cold environments upon tumor growth, it was decided to select a single methylcholanthrene-induced tumor and by syringe inoculation keep the tumor type and dose factors constant. Sarcoma Me–1, which had a typical, rapid, nonulcerated course was accordingly selected from the animals in table 1, experiment 1. Its malignancy was clearly demonstrated by some 35 successive intramuscular transplantations into Bar Harbor strain C3H mice over a period of 2 years, with almost 100 percent takes. Dr. Harold Stewart, of the National Cancer Institute, examined the tumor during the experimental period and diagnosed it as a fibrosarcoma of subcutaneous tissue. Figure 1 is a high-power view of a section showing the histologic character and high invasiveness of the tumor in muscle. The nuclei are large and darkly staining, and there are numerous mitotic figures. The cells have invaded the muscle bundles along connective tissue planes, and there is no suggestion of successful encapsulation.

For experimental purposes, this sarcoma had a further advantage in that when inoculated into the subcutaneum it grew at first mainly in two dimensions, as a loose, flat, coin-shaped mass, permitting exact measurement of the major and minor axes. Earle (*13*) has described a similar condition in a histologic study of subcutaneous versus intramuscular inoculations of both Jensen and Walker tumors. Until the axes measured 20 to 25 mm., the sarcoma did not become adherent to the underlying muscle fascia. Thus by folding up the skin the thickness could also be measured with a pair of calipers. In one experiment (table 3) following 30 days of tumor growth, the animals were all killed, and the sarcomas dissected out and measured directly. The product of the two axes measured at autopsy agreed closely with external measurements of the tumor made on the same day and gave us great confidence in the accuracy of our external measurements.

The plan of the experiment was similar to that with methylcholanthrene.

Three-months-old C3H male and female litter mates were equally divided between hot and cold rooms and acclimatized for 1 month. A suspension of sarcoma Me–1 was then injected and tumor growth followed by palpation and external measurement once or twice a week. An effort was made to standardize the preparation of the suspension and the injection technique. On the day of inoculation, tumor-bearing animals were exsanguinated, thoroughly scrubbed, put through alcohol and bichloride, and the sarcoma was aseptically exposed in a sterile operating room. Slices of tumor were then removed, weighed, put through a fine Monel tissue press (perforations $\frac{1}{64}$ inch) and suspended in sufficient sterile oxygenated Ringer-Locke solution [5] to make a 10-percent suspension. After vigorous shaking, the mixture was strained through a 50-mesh Monel sieve and quickly loaded

[5] *NaCl, 8.5 gm; KCl, 0.3 gm; $NaHCO_3$, 0.2 gm; dextrose, 1.0 gm; $H_2O$, 750 cc.; phenol red indicator. The solution was adjusted to acid by bubbling in $CO_2$, 0.2 gm. of $CaCl_2$ diluted and agitated was added and the pH adjusted to 7.4 by bubbling in $O_2$ and the solution brought to 1,000 cc. It was then sterilized by pressure through a Seitz filter.*

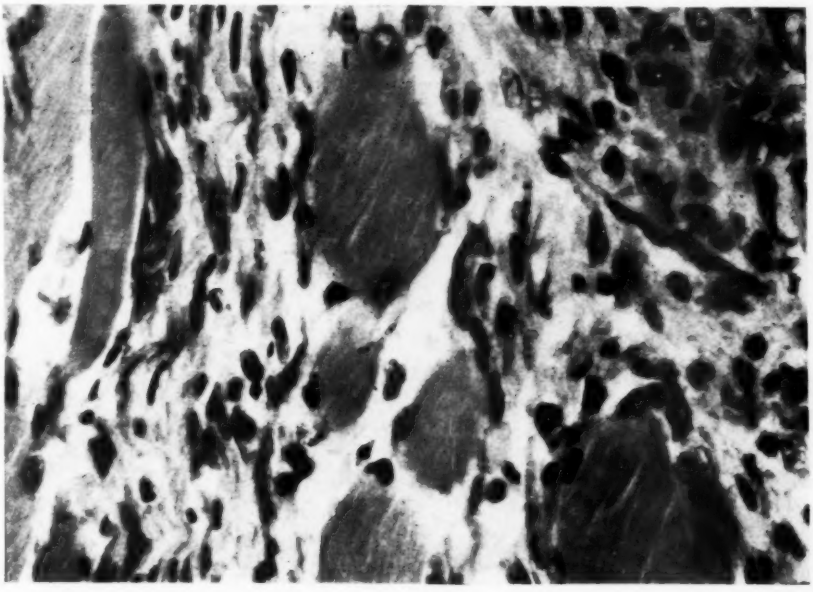

FIGURE 1.—Mouse sarcoma Me–1, showing histologic detail and invasive character when implanted into muscle. × 450.

into sterile 1-cc. tuberculin syringes fitted with 1-inch, 23-gage needles. Inoculations were made in an adjoining service room by using an antiseptic technique of soap, alcohol, bichloride, injection, alcohol, and a final swab of melted petrolatum. As it took about 2 hours to transplant the sarcoma, the animals in the hot and cold rooms were injected alternately in order to keep constant the factor of possible deterioration of tumor cells. However, in a separate experiment using the same method, in which the suspension was allowed to stand 6 hours in syringe before injection, the takes were 100 percent. The dose used for each mouse was 0.25 cc. Great care was exercised to prevent settling out of tumor cells. Before injection a bubble of air was aspirated into the syringe, and by inverting and gently shaking, homogeneity of the suspension was assured.

## SUBCUTANEOUS-INJECTION EXPERIMENT

Table 2 summarizes the results of our first experiment upon the growth of sarcoma Me–1 transplanted subcutaneously into hot- and cold-room animals. The table shows clearly that in spite of the initial high percentage of takes, after about the eighteenth day there was a rapid and progressive regression of all but 16 percent of the tumors in the cold room while 79 percent were actively growing in the hot room on the forty-sixth day. Since the subcutaneous sarcomas grew mainly in two dimensions during the experimental period, one can calculate fairly accurately the area of each by multiplying the product of the axes by the factor $\pi/4$.[6] In figure 2 the data of table 2 have been so calculated, averaged, and expressed graphically. It shows in a striking way the difference in the tumor growth in 28 pairs of litter-mate C3H males.

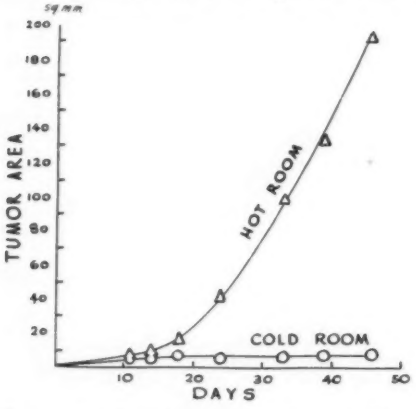


FIGURE 2.—Growth of mouse sarcoma Me–1, implanted subcutaneously in some 28 pairs of litter-mate male C3H mice divided and acclimatized to 92° and 65° F., respectively. Data from table 2.

Tables 3 and 4 give the results of the experiment on the subcutaneous growth of sarcoma Me–1 on female mice of the same strain. In order to collect quantitative data on the actual tumor weights in addition to tumor area the animals were killed on the thirtieth day following implantation. The tumors were first measured externally in the usual way, then quickly and carefully dissected out, measured directly, and weighed in a weighing bottle on an analytical balance (table 3). The results, summarized in table 4, essentially duplicated those of the previous experiment. The average weight of all sarcomas in the hot room at the end of the 30 days was 288.1 mg. as compared with only 34.2 mg. in the cold room, if one includes regressions. Although 12 days following implantation 100 percent of the animals were tumorous, 97 percent in the hot room and 48 percent in the cold room remained tumorous at the end of 30 days. Undoubtedly more tumors

[6] *Area of ellipse* $= \pi ab$, *where* a *and* b *are semiaxes.*

TABLE 2.—*Growth of mouse sarcoma Me–1 in 4-months-old litter-mate male C3H mice, injected subcutaneously with 0.25 cc. of a 10-per cent suspension of the sarcoma into the right lumbar area*

| Environment | Time after inoculation | Number of animals | Positive | Negative | Tumors | Average product of major times minor axis | | Corr. average area (positives) | Corr. average area (total) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Positives | Total | Average positives times $\pi/4$ | Average total times $\pi/4$ |
| | *Days* | | *Number* | *Number* | *Percent* | | | *Square millimeters* | *Square millimeters* |
| Hot room | 11 | 28 | 27 | 1 | 96 | 9.1 | 8.8 | 7.1 | 6.9 |
| | 14 | 28 | 27 | 1 | 96 | 11.7 | 11.3 | 9.2 | 8.9 |
| | 18 | 28 | 27 | 1 | 96 | 21.8 | 21.0 | 17.1 | 16.5 |
| | 24 | 28 | 25 | 3 | 89 | 58.0 | 51.8 | 45.5 | 40.7 |
| | 33 | 26 | 21 | 5 | 81 | 194.0 | 156.6 | 152.4 | 123.0 |
| | 39 | 24 | 19 | 5 | 79 | 214.4 | 169.6 | 168.3 | 133.3 |
| | 46 | 24 | 19 | 5 | 79 | 314.5 | 249.0 | 247.0 | 195.6 |
| Cold room | 11 | 35 | 31 | 4 | 89 | 6.9 | 6.1 | 5.4 | 4.8 |
| | 14 | 35 | 32 | 3 | 91 | 8.5 | 7.8 | 6.7 | 6.1 |
| | 18 | 35 | 21 | 14 | 60 | 14.5 | 8.7 | 11.4 | 6.8 |
| | 24 | 35 | 15 | 20 | 43 | 16.8 | 7.2 | 13.2 | 5.6 |
| | 33 | 33 | 8 | 25 | 24 | 34.0 | 8.2 | 26.7 | 6.4 |
| | 39 | 32 | 6 | 26 | 19 | 47.8 | 9.0 | 37.5 | 7.1 |
| | 46 | 31 | 5 | 26 | 16 | 75.8 | 12.2 | 59.5 | 9.6 |

would have regressed in the cold room if the experiment had been allowed to go 46 days as did the previous experiment. The details of measurement are included in table 3 in order to show the actual course of the regression. The accuracy of the external tumor measurements can be observed by comparing them with direct tumor measurements taken on the same day at autopsy. The average product of the axes measured externally on the thirtieth day in hot-room animals was 135.6 mm.$^2$ as compared with direct measurements at autopsy averaging 133.8 mm.$^2$. Similar figures in cold-room animals are 20.9 mm.$^2$ and 20.5 mm.$^2$.

The average tumor areas of table 4, calculated from the measured axes (table 3) are plotted in figure 3. Again the increase in average tumor area in hot-room animals strikingly exceeds that in cold-room animals. The figure, however, does not show, as does the table, the number and extent of actual tumor regressions.

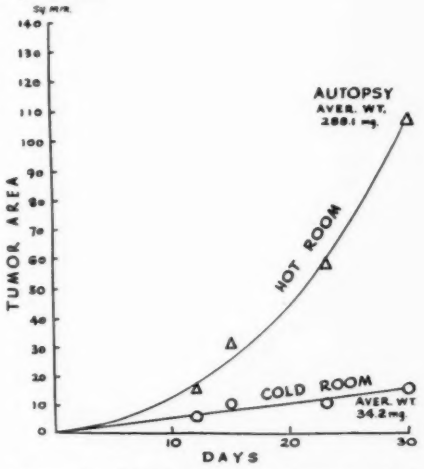


FIGURE 3.—Growth of mouse sarcoma Me–1, implanted subcutaneously in 25 pairs of litter-mate female C3H mice, divided and acclimatized to 92° and 65° F., respectively. Data from table 4.

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TABLE 3.—*Growth of mouse sarcoma Me–1 in 4-months-old litter-mate female C3H mice, injected subcutaneously with 0.25 cc. of a 10-percent suspension of the sarcoma into the right lumbar area*

| Environment | Animal No. | Product of axes width × length at various periods after inoculation (in days) | | | | | Weight of tumor at autopsy |
|---|---|---|---|---|---|---|---|
| | | 12 | 15 | 23 | 30 | | |
| | | | | | Before autopsy | At autopsy | |
| | | | | | | | *Milligrams* |
| Hot room | 1100 | 9 | 16 | 64 | 72 | 81 | 100 |
| | 1127 | 4 | 28 | 96 | 216 | 180 | 290 |
| | 1311 | 9 | 66 | 130 | 260 | 285 | 554 |
| | 1598 | 16 | 42 | 143 | 168 | 154 | 372 |
| | 1601 | 9 | 36 | 42 | 104 | 104 | 132 |
| | 1674 | 15 | 48 | 72 | 150 | 144 | 305 |
| | 1751 | 45 | 70 | 154 | 260 | 294 | 580 |
| | 1758 | 9 | 16 | 36 | 49 | 56 | 170 |
| | 1796 | 45 | 54 | 80 | 120 | 143 | 262 |
| | 1805 | 77 | 150 | 195 | 400 | 374 | 1,200 |
| | 1826 | 25 | 64 | 88 | 180 | 180 | 330 |
| | 1860 | 45 | 54 | 117 | 221 | 160 | 435 |
| | 1020 | 16 | 35 | 99 | 176 | 180 | 362 |
| | 1076 | 4 | 9 | 20 | 25 | 30 | 42 |
| | 1156 | 8 | 24 | 50 | 77 | 84 | 165 |
| | 1288 | 25 | 30 | 30 | 30 | 20 | 23 |
| | 1298 | 9 | 4 | 0 | 0 | 0 | 0 |
| | 1334 | 12 | 35 | 72 | 156 | 120 | 239 |
| | 1348 | 1 | 4 | 9 | 12 | 15 | 19 |
| | 1388 | 8 | 28 | 54 | 108 | 105 | 217 |
| | 1451 | 25 | 30 | 40 | 70 | 88 | 118 |
| | 1606 | 18 | 45 | 144 | 286 | 308 | 799 |
| | 1657 | 4 | 9 | 16 | 9 | 8 | 8 |
| | 1669 | 25 | 48 | 120 | 221 | 238 | 525 |
| | 1670 | 9 | 9 | 9 | 6 | 6 | 5 |
| | 1675 | 4 | 9 | 12 | 8 | 8 | 5 |
| | 1697 | 30 | 36 | 64 | 132 | 120 | 233 |
| | 1717 | 24 | 88 | 117 | 266 | 255 | 615 |
| | 1747 | 24 | 40 | 70 | 150 | 140 | 250 |
| Total | | 554 | 1,127 | 2,143 | 3,932 | 3,880 | 8,355 |
| Average | | | | | | | 288.1 |
| Average (positives) | | | | | | | 296.6 |
| Cold room | 1102 | 9 | 9 | 1 | 0 | 0 | 0 |
| | 1125 | 4 | 4 | 0 | 0 | 0 | 0 |
| | 1312 | 9 | 4 | 0 | 0 | 0 | 0 |
| | 1597 | 9 | 32 | 40 | 77 | 70 | 125 |
| | 1602 | 4 | 4 | 4 | 0 | 0 | 0 |
| | 1673 | 9 | 16 | 15 | 12 | 12 | 14 |
| | 1752 | 16 | 24 | 20 | 9 | 9 | 12 |
| | 1759 | 9 | 25 | 35 | 63 | 63 | 65 |
| | 1795 | 16 | 36 | 42 | 80 | 81 | 200 |
| | 1806 | 1 | 4 | 0 | 0 | 0 | 0 |
| | 1825 | 25 | 42 | 30 | 30 | 30 | 57 |
| | 1862 | 4 | 1 | 0 | 0 | 0 | 0 |
| | 1137 | 1 | 16 | 77 | 135 | 126 | 175 |
| | 1211 | 1 | 4 | 0 | 0 | 0 | 0 |
| | 1264 | 1 | 4 | 1 | 0 | 0 | 0 |
| | 1399 | 4 | 4 | 4 | 4 | 4 | 6 |
| | 1424 | 4 | 4 | 16 | 20 | 20 | 25 |
| | 1434 | 4 | 4 | 4 | 0 | 0 | 0 |
| | 1456 | 9 | 16 | 4 | 0 | 0 | 0 |
| | 1519 | 4 | 9 | 4 | 0 | 0 | 0 |
| | 1661 | 16 | 12 | 9 | 0 | 0 | 0 |
| | 1668 | 4 | 4 | 4 | 6 | 9 | 10 |
| | 1684 | 4 | 16 | 12 | 0 | 0 | 0 |
| | 1696 | 4 | 25 | 25 | 80 | 80 | 156 |
| | 1741 | 4 | 4 | 4 | 6 | 8 | 11 |
| Total | | 175 | 323 | 351 | 522 | 512 | 856 |
| Average | | | | | | | 34.2 |
| Average (positives) | | | | | | | 65.8 |

TABLE 4.—*Growth of mouse sarcoma Me–1 in 4-months-old litter-mate female C3H mice, injected subcutaneously with 0.25 cc. of a 10-percent suspension of the sarcoma into the right lumbar area (data from table 3)*

| Environment | Time after inoculation | Number of animals | Positive | Negative | Tumors | Average product of axes, width times length | | Corr. average area (positives) | Corr. average area (total) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Positives | Total | Average positives times $\pi/4$ | Average total times $\pi/4$ |
| | *Days* | | *Number* | *Number* | *Percent* | | | *Square millimeters* | *Square millimeters* |
| Hot room | 12 | 29 | 29 | 0 | 100 | 19.1 | 19.1 | 15.0 | 15.0 |
| | 15 | 29 | 29 | 0 | 100 | 38.9 | 38.9 | 30.6 | 30.6 |
| | 23 | 29 | 28 | 1 | 97 | 76.5 | 73.9 | 60.1 | 58.0 |
| | [1] 30 | 29 | 28 | 1 | 97 | 140.4 | 135.6 | 110.3 | 106.5 |
| | [2] 30 | 29 | 28 | 1 | 97 | 138.6 | 133.8 | 108.9 | 105.2 |
| Cold room | 12 | 25 | 25 | 0 | 100 | 7.0 | 7.0 | 5.5 | 5.5 |
| | 15 | 25 | 25 | 0 | 100 | 12.9 | 12.9 | 10.1 | 10.1 |
| | 23 | 25 | 20 | 5 | 80 | 17.5 | 14.0 | 13.7 | 11.0 |
| | [1] 30 | 25 | 12 | 13 | 48 | 47.5 | 20.9 | 37.3 | 16.4 |
| | [2] 30 | 25 | 12 | 13 | 48 | 46.5 | 26.5 | 36.5 | 16.1 |

[1] Before autopsy. [2] At autopsy.

## INTRAMUSCULAR-INJECTION EXPERIMENT

In another series of C3H mice, sarcoma Me–1 was implanted intramuscularly in in the right thigh. The same methods of selection of animals, acclimatization, diet, preparation of suspension, etc., were used as before, the only difference being that the sarcoma was injected into the muscle. Table 5 (experiment 1) summarizes the findings on 30 pairs of male mice. Although the percentage of takes was high, there were relatively no regressions in the cold-room animals. Not only that, but the sarcomas seemed to grow almost as readily in the cold as in the hot room, the average product of axes being 189.0 and 274.5, respectively. It was, of course, much more difficult to measure the intramuscular sarcomas, due to the fact that during the initial stages they were deeply imbedded in, and hidden by surrounding muscle, bone, etc. Furthermore, the growth was in three dimensions, and although the mass was at first spherical, it soon took on an elongated, multilobular shape as it invaded available surrounding muscle. It was thus impossible to obtain accurate measurements of the three major axes, so only the two largest were taken (usually length and width) in order to obtain approximate data on sarcoma growth. It was likewise impossible to obtain quantitative data upon sarcoma weights, since the tumor was so invasive when implanted into muscle as to make it difficult to dissect quantitatively from surrounding tissue. The data in table 5, experiment 1, are therefore not of the same order of accuracy as in the case of the subcutaneous tumors. However, the data show that sarcoma Me–1 readily grows in cold-room animals when implanted intramuscularly and with practically no regressions.

This experiment was repeated in identical fashion with female mice (table 5, experiment 2). Here again the conclusion is evident that sarcoma Me–1 grows about equally well at both temperatures when implanted intramuscularly.

## DISCUSSION

That subcutaneous tumors arise and grow faster in the hot room than in the cold room, when chemically induced and subsequently transplanted, seems clearly demonstrated. The interpretation of this result, however, is difficult.

TABLE 5.—*Growth of mouse sarcoma Me–1 in 4-months-old C3H mice injected intramuscularly with 0.25 cc. of a 10-percent suspension of the sarcoma into the right thigh*

EXPERIMENT 1

| Environment | Sex | Time after inoculation | Positive | Negative | Tumors | Average product of two major axes | |
|---|---|---|---|---|---|---|---|
| | | | | | | Positives | Total |
| | | *Days* | *Number* | *Number* | *Percent* | | |
| Hot room | M | 12 | 28 | 2 | 93.3 | 38.4 | 35.8 |
| | | 15 | 28 | 2 | 93.3 | 65.9 | 61.5 |
| | | 23 | 28 | 2 | 93.3 | 163.5 | 152.6 |
| | | 30 | 27 | 2 | 93.1 | 294.8 | 274.5 |
| Cold room | M | 12 | 25 | 5 | 83.3 | 38.0 | 31.7 |
| | | 15 | 30 | 0 | 100.0 | 51.1 | 51.1 |
| | | 23 | 29 | 0 | 100.0 | 113.5 | 113.5 |
| | | 30 | 24 | 1 | 96.0 | 196.8 | 189.0 |

EXPERIMENT 2

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Hot room | F | 7 | 18 | 0 | 100.0 | 26.2 | ..... |
| | | 10 | 18 | 0 | 100.0 | 85.1 | ..... |
| | | 14 | 18 | 0 | 100.0 | 147.4 | ..... |
| | | 17 | 17 | 0 | 100.0 | 214.4 | ..... |
| | | 21 | 17 | 0 | 100.0 | 263.1 | ..... |
| Cold room | F | 7 | 22 | 0 | 100.0 | 29.6 | ..... |
| | | 10 | 22 | 0 | 100.0 | 63.5 | ..... |
| | | 14 | 21 | 0 | 100.0 | 101.6 | ..... |
| | | 17 | 21 | 0 | 100.0 | 136.1 | ..... |
| | | 21 | 21 | 0 | 100.0 | 206.9 | ..... |

Since both rooms were constantly illuminated by a 60-watt Mazda bulb, the light factor was the same. Also practically no ultraviolet radiation is emitted from this type of bulb and, therefore, could have no influence on the results.

Sex differences do not account for our findings since animals of both sexes gave the same results. Also such factors as genetic constitution, age, and technical operations of tumor preparation, dose, and injection were carefully controlled, and they were the same for both hot- and cold-room animals.

The diet appeared not to be a limiting factor in tumor growth. To test this, 20 pairs of litter-mate C3H adult mice of both sexes were divided between the 2 rooms and placed in individual metabolism cages suitable for measuring food consumption. A fortified Sherman diet was then given each animal ad libitum, and the daily food consumption was recorded. Over a period of 30 days (after acclimatization), the average weight of food consumed by the 20 mice in the cold room was 2.7 times that consumed by their litter mates in the hot room. In another experiment paired feeding was instituted in a group of 14 pairs of litter mates. Here the food for a cold-room animal was daily measured out and determined by the food consumption of its hot-room litter mate on the preceding day. It quickly became apparent that the food consumed by the hot-room animals was not even a maintenance diet for their cold-room litter mates, as they were all dead within 3 weeks. Nevertheless, in spite of the relatively low caloric intake of hot-room animals, the diet was still more than adequate for tumor growth since the tumors not only grew, but grew faster than in the cold-room animals.

Various investigators (*14*, *15*) have found that the basal metabolic rate of animals is sharply higher when the animals are adapted to cool environments than to warm. However, our results failed to

show any direct connection between general body metabolism and growth rate of transplantable tumors. Intramuscular inoculation resulted in growth rates about equally rapid in both hot- and cold-room animals. With subcutaneous inoculation, however, tumor growth was very much more rapid in hot-room animals; also tumors induced by subcutaneous injection of methylcholanthrene arose significantly earlier under these same tropical conditions.

The injection site is evidently an important factor. The warm environment may, of course, have a direct effect upon the tumor cells. It is possible that the skin and fur of the animals do not completely insulate the tumor cells, subcutaneously implanted, from the external temperatures of the surrounding air. This explanation is suggested by the work of Smith and Fay. It is planned to test this by inserting a needlelike thermocouple into various regions of animals subjected to our experimental conditions. On the other hand it is well-known that the mammalian body tends to conserve heat loss in a cold environment by relative peripheral vasoconstriction. Conversely, in a tropical environment heat loss would be increased by a relative peripheral vasodilatation. It is possible then that the richer peripheral blood supply in the hot-room animals and, perhaps, increased local skin metabolism favors tumor growth and genesis.

## SUMMARY

Subcutaneous methylcholanthrene-induced tumors, under carefully controlled conditions, arise earlier in C3H mice kept in a constant tropical environment (92° F., 50 percent relative humidity) than in litter mates kept in a constant temperate environment (65° and variable humidity). In 2 experiments the average latent period was $9.78 \pm 0.17$ weeks for 45 C3H mice in the hot room as compared with $11.70 \pm 0.22$ weeks for 52 C3H mice in the cold room. The observed difference, 1.92 weeks, is $>$ 6 times the standard error of the difference and, therefore, highly significant.

A single methylcholanthrene-induced transplantable spindle-cell sarcoma was quantitatively injected both subcutaneously and intramuscularly into strain C3H mice maintained in hot and cold rooms. When injected subcutaneously, the sarcoma grew rapidly in the hot-room animals and only slowly or actually regressed in the cold-room animals. When injected intramuscularly the sarcoma grew equally well in both hot- and cold-room animals.

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# Photometric Histochemical Determination of Thymonucleic Acid in Experimental Epidermal Carcinogenesis[1]

By R. E. STOWELL, *research associate, Barnard Free Skin and Cancer Hospital, and research fellow in cytology, Washington University, St. Louis* [2]

This estimation of thymonucleic acid by the photometric determination of the Feulgen "nucleal" reaction is a part of a concerted effort of the research staff of the Barnard Free Skin and Cancer Hospital, under the direction of Dr. E. V. Cowdry, to measure alterations in the cells of a graded series of specimens of mouse skin treated with methylcholanthrene. Studies of alterations in nuclear viscosity (*1*), changes in cellular, nuclear, and nucleolar sizes (*2*), determinations of mitotic frequency (*3*), and microincineration studies (*4*) have already been published, and chemical investigations of the ascorbic acid, iron, and lipid content of the mouse epidermis are in press (*5, 6*). In the present paper a comparison is made of the thymonucleic acid content of (1) the normal epidermis of the mouse skin, (2) epidermis which has become slightly differentiated or hyperplastic after painting with pure benzene and with 0.6 percent methylcholanthrene in benzene, (3) papillomas, and (4) carcinomas following prolonged treatment with the same carcinogen.

During the past 2 years a photoelectric microphotometer has been developed in this hospital which is suited for quantitative measurements of stain in tissue sections. If the stain used is specific for one tissue component, that component may be measured indirectly by determining the absorption of complementary monochromatic light by the stain. Since the Feulgen reaction for thymonucleic acid is one of the most specific of histologic stains, it is suitable for such photometric study.

There has been some controversy regarding the degree of specificity of the Feulgen reaction for thymonucleic acid. Some writers, including Semmens (*7*), have attacked the claims of specificity, but Barber and Price (*8*) have refuted Semmens' claims concluding that the reaction is specific for potential aldehyde groups of chromatin. Wyckoff, Ebeling, and Ter Louw (*9*) found that ultraviolet photomicrographs were essentially similar to the results with Feulgen stained material. Caspersson and Schultz (*10–12*) used the Feulgen stain for confirmation of the presence or absence of desoxyribose nucleic acid (thymonucleic acid) in connection with their measurements with ultraviolet light. It seems that the staining is chiefly due to thymonucleic acid. Our work is based on the assumption that stained parts of the tissue are thymonucleic acid and that the amount of stain is proportional to the amount of thymonucleic acid present in the tissue.

Masayama and Yokoyama (*13*), using a colorimetric macrochemical technique, reported an increase in thymonucleic acid in cancer of the liver of rats which had been fed *p*-dimethylaminoazobenzene. Numerous workers have made visual observations on cancer cells after using the Feulgen technique. Cowdry (*14*), comparing

[1] *Aided by a grant from an anonymous donor.*

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tissue cultures of rat sarcomas and of normal fibroblasts, reported an increased amount of Feulgen stained material in the sarcoma cells. Ludford (*15*), working with tar cancer, and Eckert and Cooper (*16*), studying squamous-cell carcinoma of the cervix, did not observe significant differences in the amount of Feulgen stain in malignant and normal cells. Additional papers are cited in the bibliography by Milovidov (*17*) who lists 450 references of work with the Feulgen technique prior to 1938.

## MATERIALS AND METHODS

Young mice of the New Buffalo strain were employed in these studies.[3] Untreated normal skin was obtained from five mice. Solutions of benzene and of 0.6 percent 20-methylcholanthrene in benzene were applied to the backs of the mice in a uniform manner by a single stroke of a No. 4 camel's-hair brush. Two groups of five mice each were painted four times in 10 days with pure benzene and killed 2 days after the last painting. Five mice were painted twice with a 0.6-percent solution of methylcholanthrene in benzene. The second painting was given 3 days after the first, and the mice were killed 7 days after the second painting. Five mice were painted nine times in 22 days with the same carcinogenic solution and killed 2 days after the last painting. Other mice which had been painted with methylcholanthrene thrice weekly for 3 months were killed at variable lengths of time following the development of carcinomas and papillomas.

The specimens of skin were cut transversely from the midpart of the back, spread on mica plates, and fixed for 18 hours in equal parts of 95 percent ethyl alcohol and a saturated aqueous solution of mercuric chloride, as suggested by Cowdry (*18*). The fixed skin was left several days in 70 percent ethyl alcohol, then dehydrated through a series of alcohols, cleared in chloroform, and infiltrated with 56° paraffin. Although the tissues were dehydrated and embedded in three different lots, all tissues received comparable treatment. Serial sections of 10$\mu$ thickness were cut from all blocks on the same day, and paraffin ribbons were straightened on a hot plate kept at 40° ± 0.5° C. The normal skins were cut obliquely to the surface to increase the area of the epidermis available for measurement.

Sections of all specimens were first stained with Harris' haematoxylin and eosin. The response of the epidermis to pure benzene and to the applications of methylcholanthrene varied considerably, and only the specimens with an epidermis more than 3 cells thick showed areas suitable for measurements with this apparatus. Five of the five specimens of normal skin, 2 of 10 specimens of skin painted with benzene, 3 of the 5 skins treated twice with methylcholanthrene, 5 of the 5 specimens painted 9 times with methylcholanthrene, 4 papillomas, and 6 carcinomas were selected for measurement.

Two, and in some cases four, sections adjacent to those stained with haematoxylin and eosin were mounted on microscope slides of measured thickness. These slides were of relatively uniform thickness, measuring 1.112–1.117 mm. Likewise the cover glasses used were carefully selected, ranging in thickness from 0.160 to 0.165 mm. Only one section of the same specimen was mounted on any one slide, but four to six sections of different specimens were arranged on each slide. Three sets of slides for each

[3] *The skin was obtained at necropsy from mice painted for other experiments by my colleague, Dr. V. Suntzeff, who granted me the privilege of removing thin slices of tissue for this study.*

arrangement of sections were prepared containing the adjacent serial sections. Two of these sets of slides were stained for thymonucleic acid, and the third control set was subjected to the same solutions with the exception of the one solution containing the basic fuchsin stain. All slides were stained simultaneously in the same dish.

The staining procedure employed was a modification of the Feulgen techniques described by de Tomasi (*19*) and Coleman (*20*). The hydrolysis in 1N HCl was altered so that it might be more exactly controlled. On the basis of preliminary observations, the hydrolysis was conducted for 20 minutes at 50° C. The stained sections were thoroughly washed in water, dehydrated, cleared in xylol, and mounted in 60 percent clarite in xylol. According to Groat (*21*) clarite is absolutely neutral; hence, it should not promote fading of most stains. It also absorbs less visible light than balsam (*22*). The mounted slides were allowed to stand 3 days before photometric measurements were begun. Slides were stored in the dark except when actual measurements were being made. The series of measurements was completed within 1 month from the time the slides were stained.

The microphotometric apparatus used is shown in figure 1. It consisted of a lamp, a microscope, a photocell, and amplification and recording apparatus.

The light source was a 50-c. p. automobile headlamp operated by storage batteries. It was mounted in a Spencer (No. 367) lamp case. A heat-absorbing filter (Corning Aklo No. 396) and a green gelatin filter (Wratten No. 58) were mounted in the filter holder. Critical illumination of the Kohler type was used.

The Spencer microscope was equipped with a × 44 achromatic objective attached to a centering nosepiece, with a × 15 compensating eyepiece and with a mechanical stage (Spencer No. 1540, type x). The slide with the stained tissue was mounted on this stage. In the eyepiece of the microscope, a rectangular diaphragm was inserted to limit the stage field to an area of 50$\mu$ by 35$\mu$.

The light from the microscopic eyepiece fell upon the target of a RCA 929 vacuum phototube. The photocell was enclosed

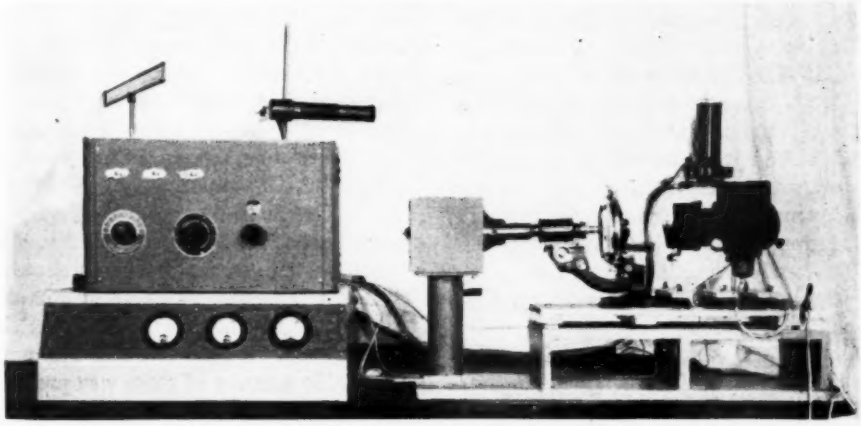

FIGURE 1.—Apparatus used for photometric measurements on sections of tissue.

in a light-tight box, from which a side tube extended over the tube of the microscope. A movable mirror was mounted in the side tube in such a way that it could be interposed in the optical system to permit visual inspection of the exact field to be measured. The mirror was then turned to permit the passage of light from the microscope to the photocell.

The photocell current was amplified by a General Electric FP54 tube incorporated in a Barth circuit [4] (*23*) with a $10^{10}$-ohm grid resistor. The amplifier output was opposed by a Leeds & Northrup student type potentiometer and the null point read with a Leeds & Northrup type R galvanometer (list No. 2500-b) and scale. Readings were made from the potentiometer after allowing a constant amount of time for the establishment of equilibrium.

At the beginning and end of each set of measurements, as well as at frequent intervals, readings were made of the light transmittance through the blank portion of the slide adjacent to the tissue being measured. In this way a correction was supplied for slight variations from decrease in light owing to battery discharge, change of amplification or potentiometer batteries, or alterations in the thickness of cover glasses, slides, or mounting media. These blank readings were plotted so that a figure for the light transmission, $I_T$, was obtained for each reading, $I_O$, made upon the stained section. The apparatus was adjusted so that $I_T$ was approximately the same at the start of each group of readings. The absorption of the roughly monochromatic green light by the reddish-purple stained thymonucleic acid was calculated by subtracting $I_O$ from $I_T$.

The mean percentage absorption for each stained tissue section was calculated as $\frac{\text{mean } I_T - \text{mean } I_O}{\text{mean } I_T} \times 100$. To correct for the absorption of light by the unstained portions of the tissue, readings were made in a similar manner upon the control slides which had not been placed in the staining solution. The mean percentage absorption of light by the stained thymonucleic acid was obtained by subtracting the mean percentage absorption of the unstained tissue from the mean percentage absorption of the stained tissue. As an indication of the variation in absorption between different areas of the section, the coefficient of variability was calculated for each set of measurements.

When possible a minimum of 50 adjacent areas were measured on each section. With the sections of normal skin and with some of the benzene-painted skins, less than 50 areas were suitable for measurements. The results of these smaller groups of measurements were averaged to obtain a more significant figure. Areas showing keratin, parakeratin, hair shafts or follicles, inflammatory cells, dermis, or artifacts were not measured. On rare occasions during the course of the measurements the sections became out of focus. They were then refocused to the center of their focal depth and the appropriate blank readings made to correct for the change in light transmittance. Since the green color of the filtered light was complementary to the color of the stain, the stained portions of the chromatin absorbing light appeared black. Stain was visible only in the chromatin of the nuclei, and there was no reason to feel that any appreciable amount of stain was present in other parts of the sections.

Certain measurements were made to

[4] *Dr. T. Enns, formerly of the Department of Physics, Washington University, rendered technical assistance in the construction of the Barth amplification circuit used in the photometric apparatus. My colleague, Lester Wicks, made helpful suggestions.*

determine the accuracy of the apparatus under the conditions of this experiment. Repeated absorption measurements on the same individual area of tissue within relatively short periods of time usually checked within less than ± 0.1 percent. On the basis of the results shown in table 1 and of other measurements it was concluded that the mean percent absorption on groups of 50 measurements on the adjacent sections of the same tissue stained simultaneously varied less than 10 percent of the calculated absorption.

## RESULTS

The results of the absorption measurements given in table 1 are summarized in table 2. These measurements show a decrease in the mean thymonucleic acid content per unit volume of skin after painting with benzene and with a solution of methylcholanthrene in benzene. Because of the great variability in the absorption measurements between different specimens of carcinomas and papillomas, mean absorption figures were not computed (table 2).

TABLE 1.—*Relative thymonucleic acid content per unit volume as measured by light absorption of various tissues stained by the Feulgen reaction*

| Tissue No. | Description | Date measured | Areas measured in each section | Absorption | Mean absorption | Coefficient of variation of areas | Mean coefficient of variation of areas |
|---|---|---|---|---|---|---|---|
| | | | *Number* | *Percent* | *Percent* | | |
| 1 | Normal skin | | [1] 76 | | 18.8 | | 21.1 |
| 2–4 | do | | [2] 96 | | 18.0 | | 27.5 |
| 5 | Skin painted 4 times with benzene | April 15 | 50 | 19.7 | 17.8 | 12.3 | 14.6 |
| | | April 29 | 52 | 16.8 | | 16.4 | |
| | | May 6 | 51 | 16.9 | | 15.0 | |
| 6 | do | April 29 | [3] 20 | | 16.7 | | 17.2 |
| 7 | Skin painted twice with methylcholanthrene | April 15 | 52 | 15.0 | 14.9 | 18.5 | 18.7 |
| | | April 30 | 53 | 15.3 | | 24.7 | |
| | | May 7 | 51 | 14.6 | | 13.6 | |
| | | May 8 | 50 | 14.5 | | 17.9 | |
| 8 | do | April 29 | 50 | 14.7 | 14.5 | 24.5 | 25.3 |
| | | May 8 | 51 | 14.4 | | 26.1 | |
| 9 | do | April 29 | 51 | 14.3 | 14.0 | 15.9 | 13.0 |
| | | May 8 | 50 | 13.7 | | 10.0 | |
| 10 | Skin painted 9 times with methylcholanthrene | April 16 | 55 | 14.7 | 14.3 | 18.2 | 19.7 |
| | | April 20 | 51 | 14.7 | | 21.0 | |
| | | May 5 | 50 | 14.4 | | 20.1 | |
| | | May 7 | 51 | 13.3 | | 19.7 | |
| 11 | do | April 20 | 51 | 14.4 | 12.5 | 17.7 | 18.5 |
| | | May 4 | 55 | 10.7 | | 19.2 | |
| 12 | do | April 20 | 55 | 14.2 | 14.5 | 19.1 | 21.9 |
| | | May 5 | 51 | 14.8 | | 25.0 | |
| 13 | do | April 21 | 52 | 12.5 | 13.6 | 22.0 | 21.6 |
| | | May 5 | 50 | 14.8 | | 21.1 | |
| 14 | do | April 21 | 50 | 12.8 | 12.7 | 20.8 | 20.6 |
| | | May 9 | 51 | 12.7 | | 20.4 | |
| 15 | Papilloma | April 13 | 52 | 11.7 | | 29.3 | |
| 16 | Papilloma with marked hyperplasia | May 8 | 51 | 14.0 | | 44.7 | |
| 17 | Papilloma with hyperchromic nuclei | April 23 | 50 | 10.5 | 10.1 | 29.4 | 32.3 |
| | | April 24 | 50 | 9.7 | | 35.1 | |
| 18 | Papilloma with hyperchromic nuclei; adjacent to above tumor. | April 23 | 50 | 27.8 | 26.9 | 21.1 | 20.2 |
| | | April 24 | 50 | 26.0 | | 19.3 | |
| 19 | Transplanted epidermal carcinoma with abundant cytoplasm. | April 14 | 50 | 10.9 | [4] 10.9 | 21.2 | [4] 17.3 |
| | | April 14 | 50 | 10.9 | | 13.3 | |
| 20 | Carcinoma | April 13 | 59 | 16.6 | 16.3 | 19.8 | 18.4 |
| | | May 6 | 52 | 15.9 | | 16.9 | |
| 21 | Carcinoma, hyperchromic nuclei | April 13 | 55 | 23.5 | | 19.0 | |
| 22 | Carcinoma, abundant cytoplasm in some areas | April 22 | 51 | 14.7 | | 24.0 | |
| 23 | Carcinoma, small cells | April 22 | 52 | 18.6 | | 18.1 | |
| 24 | Carcinoma | April 22 | 52 | 17.7 | | 15.3 | |
| 25 | do | May 6 | 51 | 19.6 | | 17.9 | |

[1] Average data of 4 adjacent sections from 1 mouse.[5]
[2] Average data for 5 sections from 3 different mice.[5]
[3] Average data of 2 adjacent sections.[5]
[4] Average of 2 sections from adjacent blocks of same tumor.
[5] These 3 groups of data were averaged because of the small number of measurements in each group.

TABLE 2.—*Summary of relative thymonucleic acid content per unit volume as measured by the light absorption of various types of tissues stained by the Feulgen reaction*

| Type of tissue | Total sections measured | Total areas measured | Absorption | | Coefficient of variation | |
|---|---|---|---|---|---|---|
| | | | Extremes | Mean | Extremes | Mean |
| | *Number* | *Number* | *Percent* | *Percent* | | |
| Normal skin | 9 | 172 | 18.0–18.8 | 18.4 | 21.1–27.5 | 24.7 |
| Benzene-painted skin | 5 | 173 | 16.8–19.7 | 17.7 | 12.3–16.4 | 14.9 |
| Skin painted twice with methylcholanthrene | 8 | 408 | 13.7–15.3 | 14.6 | 10.0–26.1 | 18.9 |
| Skin painted 9 times with methylcholanthrene | 12 | 622 | 10.7–14.8 | 13.7 | 17.7–25.0 | 20.3 |
| Papillomas | 6 | 303 | 9.7–27.8 | | 19.3–44.7 | 29.8 |
| Carcinomas | 9 | 472 | 10.9–23.5 | | 13.3–24.0 | 18.4 |

Probably of more importance than the thymonucleic acid content per unit volume of skin is the thymonucleic acid per cell. To compare the number of nuclei per measured area with the thymonucleic acid content, mean nuclear counts were determined for certain specimens as shown in table 3. Despite large variations in thymonucleic acid per unit volume of tissue, the mean amount of thymonucleic acid was similar in the cells of most specimens. However, it did appear to be significantly increased in carcinomas 20, 21, and 24.

Carcinoma 19 was a relatively low grade methylcholanthrene-induced tumor which had been transplanted through five serial transfers by my colleague, Dr. Zola Cooper. Specimens 21, 22, and 23 were separate carcinomas from the back of one mouse. Papillomas 17 and 18, which also showed great differences in thymonucleic acid per unit volume of tissue, were adjacent tumors on one mouse.

TABLE 3.—*Comparison of mean number of nuclei and relative thymonucleic acid content, as measured by the mean light absorption, of single specimens of various types of tissue stained by the Feulgen reaction*

| Tissue No. | Description | Absorption | Average nuclei per field [1] | Mean absorption per nucleus |
|---|---|---|---|---|
| | | *Percent* | *Number* | *Percent* |
| 1 | Normal skin | 18.8 | 26 | 0.7 |
| 5 | Benzene-painted skin | 17.8 | 22 | .8 |
| 7 | Skin painted twice with methylcholanthrene | 14.9 | 21 | .7 |
| 10 | Skin painted 9 times with methylcholanthrene | 14.3 | 14 | 1.0 |
| 15 | Papilloma | 11.4 | 16 | .7 |
| 16 | do | 14.0 | 15 | .9 |
| 17 | do | 10.1 | 13 | .8 |
| 18 | do | 26.9 | 37 | .7 |
| 19 | Carcinoma | 10.9 | 10 | 1.1 |
| 20 | do | 16.3 | 12 | 1.4 |
| 21 | do | 23.5 | 14 | 1.7 |
| 22 | do | 14.7 | 15 | 1.0 |
| 23 | do | 18.6 | 21 | .9 |
| 24 | do | 17.7 | 12 | 1.5 |
| 25 | do | 19.6 | 23 | .9 |

[1] Determined by counting approximately 50 fields.

The absorption measurements on various unstained control slides showed only slight differences, varying between 0.58 and 0.72 percent.

## DISCUSSION

The obvious irregularities in microtechnical procedures were greatly reduced in this study by the careful standardization of technique. The measurements of similar areas of adjacent serial sections mounted on different slides gave comparable results since all sections were stained simultaneously in the same solutions. By using sections 10$\mu$ thick and measuring two adjacent sections, variations in measurements resulting from section thickness were decreased. The use of relatively thick sections and of areas 50$\mu$ by 35$\mu$ gave average readings for many nuclei in a definite volume of tissue and reduced the number of individual areas that should be measured to give significant results. Possible errors because of the inability of dense masses of chromatin to absorb an amount of light exactly proportional to the amount of thymonucleic acid present were reduced by the averaging of absorp-

tion measurements of hundreds, and in some cases thousands, of nuclei and by the consideration of mean measurements on any one specimen only as relative to comparable measurements on other specimens.

It is important to know to what extent fading of stain influenced the results of this experiment. In instances where measurements were repeated on adjacent sections of the same specimen the mean results for 50 area measurements decreased in 19 cases, increased in 4 cases, and remained the same in 1 case when compared with the previous measurements. Although some of these variations were explained by the measurement of different parts of the specimen, the stain did fade slightly during the course of the experiment. There was no evidence that appreciable fading occurred during the measurements on any one section. Areas of each section were measured once only. The sequence of measurements of sections was arranged so that the results of fading would be largely corrected by averaging 2 or more specimens. Hence, stain fading did not significantly alter the results of absorption measurements.

There were significant differences between various specimens receiving the same treatment as well as between different parts of the same section. The epidermis was usually thicker near the peripheral portion of the painted area. In measuring adjacent sections of the same specimen, usually no attempt was made to measure exactly the same part of each specimen. This accounts for most of the larger discrepancies shown in table 1 between the percentages of absorption of the same specimen. In those cases in which the same areas were measured, the results agreed closely.

The figures for coefficient of variability are an indication of the degree of difference in thymonucleic acid between different parts of the same section. The variability is greatest in papillomas and especially in those with marked hyperplasia. As shown in table 1, papilloma 16, which had a pronounced degree of hyperplasia, had a 44.7 percent coefficient of variation.

The normal epidermis of the mouse which is not differentiated and is only two or three cells thick contains more thymonucleic acid per unit of volume than epidermis treated with benzene or methylcholanthrene. These reagents both promote a differentiation or hyperplasia of the epidermis. The basal layer of the differentiated epidermis contains cells with less cytoplasm and with nuclei which stain more intensely than the cells of the spinous and granular layers. As these latter types of cells increase in number, the thymonucleic acid content of the epidermis decreases. Generally, the specimens of skin with more hyperplasia have less thymonucleic acid per unit volume. These differences are illustrated in figure 2.

Papillomas showed the greatest variation in their thymonucleic acid content and also in the coefficient of variation of the individual area measurements. The amounts of stain per area in specimens of markedly hyperplastic epidermis did not seem to follow a normal distribution curve. Figure 3, *A*, illustrates 2 adjacent papillomas which varied greatly in their absorption of light and number of nuclei per field. The papilloma on the left (No. 18) averaged 26.9 percent absorption and 37 nuclei per field, while the other papilloma (No. 17) had 10.1 percent absorption and 13 nuclei per field. The amount of thymonucleic acid per cell was similar in both tumors (table 3).

Carcinomas also exhibit great variability in content of thymonucleic acid per unit volume, as indicated in table 1 and figure 3, *B* and *C*. Figure 3, *B*, is

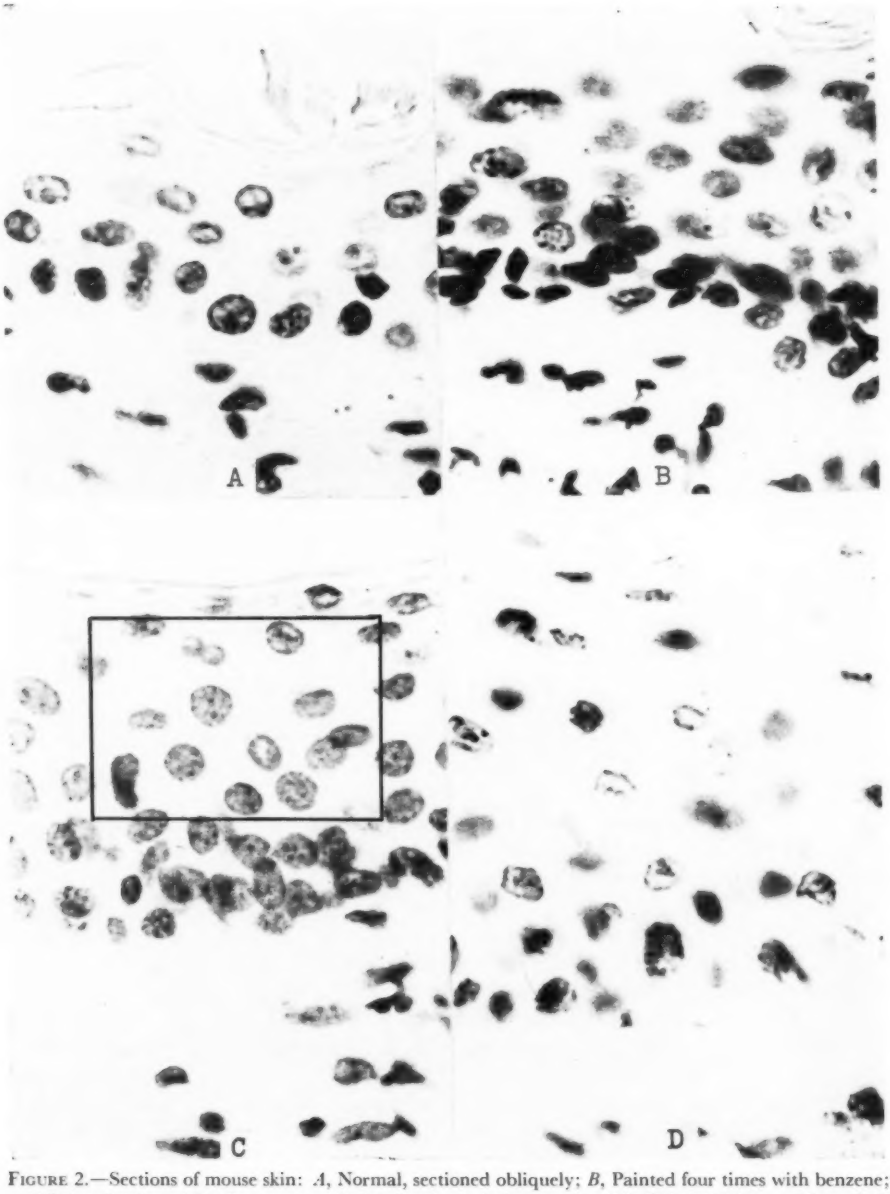


FIGURE 2.—Sections of mouse skin: *A*, Normal, sectioned obliquely; *B*, Painted four times with benzene; *C*, Painted twice with 0.6 percent methylcholanthrene in benzene; and *D*, Painted 9 times with methylcholanthrene in benzene. Size of areas measured is outlined in C. All sectioned at 10$\mu$ and stained by Feulgen reaction. × 900.

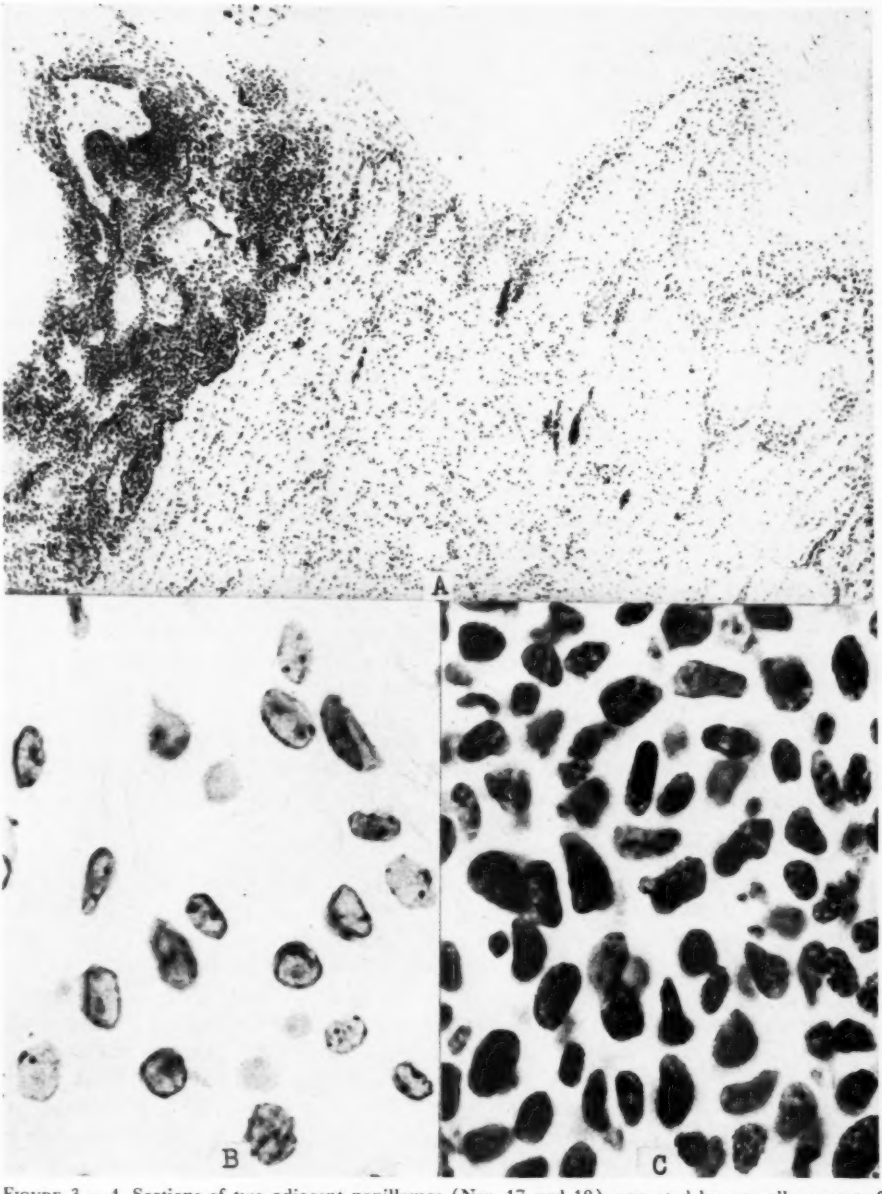


FIGURE 3.—*A*, Sections of two adjacent papillomas (Nos. 17 and 18) separated by a small amount of hyperplastic epidermis. × 83; *B*, Carcinoma 19. × 900; and *C*, Carcinoma 21. × 900. Note the differences in amount of thymonucleic acid stained by the Feulgen technique in the two papillomas and the two carcinomas. All sections at 10$\mu$.

from specimen 19, which averaged 10.9 percent absorption by thymonucleic acid, and figure 3, *C*, is from carcinoma 21, which averaged 23.5 percent absorption. The possible errors in the selection of a single specimen of a tumor as representative of carcinomas or papillomas are obvious.

The similarity in mean amount of stain per nucleus in most of the specimens listed in table 3 is of interest in connection with Mainland's statement (*24*) that there is a similar amount of basophilic chromatin in all nuclei of a specimen and that the larger the nucleus the less the amount of stained material in the given volume.

Caspersson, Schultz, and Aquilonius (*10, 25–27*) and Meglitsch (*28*) have shown that the thymonucleic acid content of a single living cell may undergo cyclic variations. However, in the present experiment variations were averaged.

Since the area shown in figure 2, *A*, is from specimen No. 1; 2, *B*, from No. 5; 2, *C*, from No. 7; and 2, *D*, from No. 10, it is interesting to compare these photographs with the results shown in table 3. For these four type specimens, it is apparent that the decrease in thymonucleic acid is accompanied by a decrease in the mean number of nuclei per unit of volume. However, variations in thymonucleic acid are not explained entirely by differences in numbers of nuclei. For instance, carcinoma 21 has 2.2 times as much thymonucleic acid as carcinoma 19 and only 1.4 times as many nuclei. Thus, in these two tumors there appears to be an actual difference in the average amount of thymonucleic acid per cell present, as represented by the figures 1.7 for carcinoma 21, and 1.1 for carcinoma 19.

There appears to be some correlation between the amount of thymonucleic acid per cell and the degree of anaplasia of the carcinomas. Additional studies on this question are now in progress. If true, this increase of thymonucleic acid is interesting in connection with the findings by Caspersson and Schultz (*10, 25, 27*) that thymonucleic acid is essential for cell division and is increased before cell division, and also with their suggestion that there is a disturbance of nucleic acid metabolism in cancer cells.

## CONCLUSIONS

A photometric apparatus is described which is satisfactory for the measurement of the amounts of stain or pigment in tissue sections. When a stain is relatively specific for one tissue component, comparative studies of that constituent may be made by determining the amounts of stain in the tissues. Such apparatus is a useful research instrument.

By using the Feulgen reaction, estimations were made of the relative amounts of thymonucleic acid at various stages of methylcholanthrene carcinogenesis in the skin of mice.

Normal epidermis showed a higher thymonucleic acid content per unit volume than skin after painting with pure benzene or with 0.6 percent methylcholanthrene dissolved in benzene. As the hyperplasia became more marked, the mean content of thymonucleic acid decreased and the variability between different areas increased.

Different specimens of carcinomas and especially of papillomas showed great differences in thymonucleic acid content per unit volume. Certain specimens of both benign and malignant tumors contained less, and others more thymonucleic acid than epidermis that was normal or had received a few treatments with the benzene solvent or carcinogen.

The mean amount of thymonucleic acid per cell was similar for most tissues, except for some carcinomas in which it was significantly increased.

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# Genetics of the Susceptibility of Mice to a Transplantable Melanoma

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The problem presented herein involves the genetic investigation of a transplantable melanoma of the mouse, S91, with special reference to the susceptibility of the $F_2$ albino hybrids of reciprocal A×dba crosses. Little (*1*) has discussed the salient points of the type of problem involved. He states (*1, p. 89*):

> The tissues of any individual are the product of a complex of genetic factors interacting with the cell substance of the egg and of its descendant cells. In a very closely inbred race, the different individuals have the same, or nearly the same complex of genes, and there is therefore no way in which any difference can be detected. In an ordinary laboratory race, the various individuals are probably characterized by different complexes many of which, however, approach each other closely, and some of which either coincide or come so near it that implants between the individuals with such complexes are as successful as autotransplants. The best place to study the inheritance of the factors which underlie tissues, whether normal or tumor, is in crosses between two closely inbred races, within each of which there is sufficient homogeneity to allow successful transplantation of the tissue in question between different individuals in approximately 100 percent of the cases attempted. In such crosses, the $F_2$ and back-cross generations give a chance to measure in terms of Mendelian units, the degree of difference between the two complexes which characterize the two parent races.

In the problem reported in this paper the importance of using inbred stocks of animals cannot be overemphasized. Accordingly, inbred strains of mice A and dba were employed. Bittner (*2*) and Murray (*3*) have reported on the breeding behavior and mammary-gland-tumor incidence of these strains, respectively. Since the A strain is practically 100 percent nonsusceptible, and the dba 100 percent susceptible to tumor S91, the two parent strains give a clear-cut difference and can be recorded as — and +, respectively.

## DESCRIPTION OF TUMOR

Cloudman has provided the following unpublished information concerning the transplantability of the melanoma S91 employed: Tumor S91 originated in a dba female at the base of the tail and was black, firm, and hairless. This tumor was first transplanted on July 12, 1937, and was not retransplanted until 8 months later. When employed in the present investigation, it was in its thirteenth transplant generation. At the present time it must be transplanted every 4 to 6 weeks. The first tissue generation contained 6 animals which were recorded as negative, but in view of the length of time required for growth at first, it is probable that at least some of these animals were killed too soon to give an adequate test of susceptibility. Including these animals, there have been only 6 percent of negatives in over 200 animals, but discarding this first experiment, the negatives are reduced to 3 percent. No regression of well-established tumors has been noted. Nearly 100 percent of animals with advanced tumors have shown pulmonary metastases at autopsy. Metastases have also been found in liver, kidney, adrenal, pan-

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creas, and many other internal locations. No growths have occurred in A stock albinos when inoculated when 4 to 8 weeks of age.

Histologically, the melanoma appears as a chaotic mass of cells staining deep blue with haematoxylin. The cells are of fairly large size and are oval to elliptical in shape. In some portions the cells may assume a spindle shape and become arranged in long cords. About the periphery of the mass the cells appear to be much smaller in size, more densely packed, and of spherical shape. The cytoplasm of the tumor cells is heavily laden with pigment granules, which generally stain a deep blue, but occasionally brown. In general, the tissue is densely packed, although in some sections it may appear somewhat loose, almost areolar, in arrangement. The intercellular substance is of a fibrous nature and stains lightly. Deposits of free melanin appear intercellularly as sometimes coarse, sometimes fine, brown granules. In general, it was found that the more densely cellular the tissue, the less free melanin was found intercellularly, although the pigment granules within the cells were just as abundant. This may indicate that the melanin is released into the interstitial tissue upon cellular breakdown. However, practically no necrotic tissue was present. In some sections, hemorrhages had occurred into the tumor tissue, in which case the interstitial tissue was permeated with extravasated blood. No phagocytosis of red blood cells by the tumor cells was observed. The enormous proliferative energy of the tumor is demonstrated by the large number of mitotic figures to be found in the sections. No histologic differences have been observed between S91 grown on strain dba,

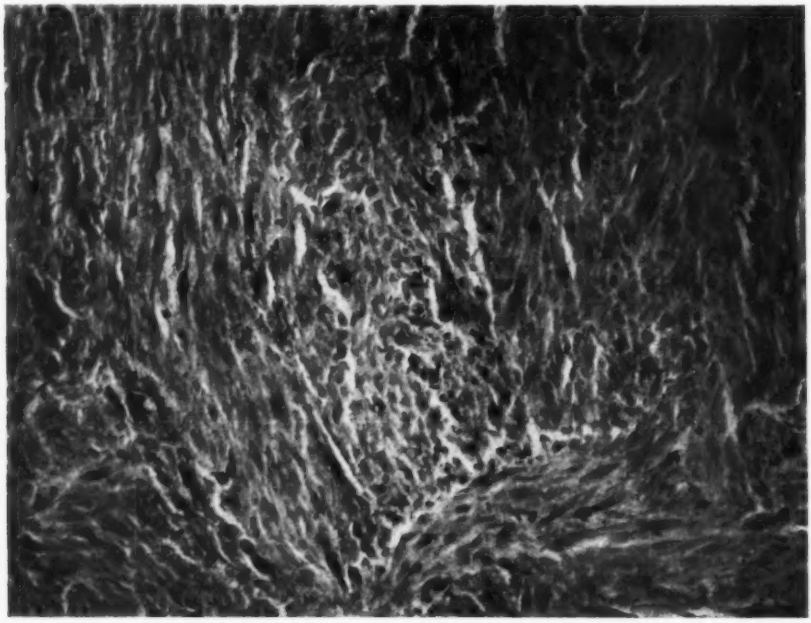

FIGURE 1.—Tumor S91, showing the pigmentation. × 200.

or on susceptible hybrids, albino or colored, of dba and A.

Figures 1 and 2 are photomicrographs of the neoplastic tissue.

## EXPERIMENTAL PROCEDURE

Pens for breeding were made up to give reciprocal crosses between the inbred A and dba strains of mice.

As soon as a female became pregnant, she was removed to a separate box. The young remained with the female until they were approximately 1 month old, at which time they were weaned, and the female was returned to the home pen. By this procedure backcrosses were avoided, since mice do not attain sexual maturity until 7 to 10 weeks of age (*4*). When the $F_1$ mice were weaned, each animal was ear-punched with a serial number, and breeding pens were made up containing one to four females and one male. Thus the $F_2$ generation produced was the result of breeding the $F_1$ individuals inter se. Extra males in the $F_1$ generation were segregated and used for inoculation purposes. The young of the $F_2$ generation, when weaned, were segregated according to sex and used for inoculation. All the mice were fed on a uniform diet of Purina dog chow.

The trocar method of inoculation was employed. A tumor about the size of a pea (4 to 8 weeks' growth) was chosen to prepare the inoculum. This tumor was carefully removed from an animal and placed in a Syracuse dish containing physiologic saline solution warmed to about 37° C. The tumor was cut into small fragments of approximately uniform size. Animals received their first inoculation at an age of from 4 to 8 weeks. This age was chosen since it is well under the age of occurrence of spontaneous tumors in the strains employed. The axillary

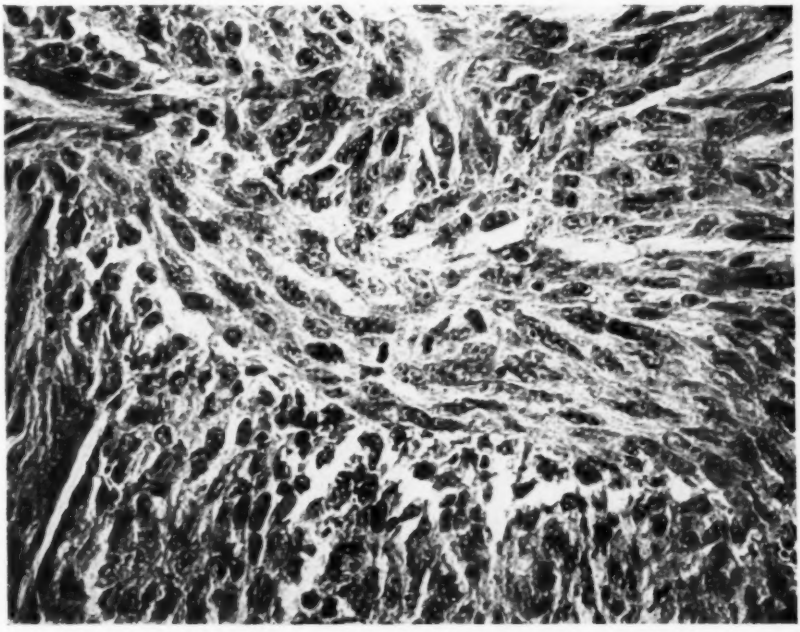

FIGURE 2.—Tumor S91, showing detailed structure. × 450.

position was selected as the inoculation site because of its rich blood supply. The trocar was inserted under the skin in the iliac region and run forward to the axillary position, where its contents were discharged. Each mouse was inoculated in the right as well as the left axilla, with few exceptions. Although growth of the transplant on one side alone is sufficient for determination of susceptibility, it was deemed wise to inoculate on both right and left sides as a check on technical errors.

A total of 38 S91 tumors were used for inoculation purposes, and all but 1 were examined histologically. Mice which had received transplants of the first 20 of these tumors but which failed to grow the transplants after 2 months or more, were reinoculated. One month after inoculation and weekly thereafter, the mice were examined for growths. Animals showing palpable growths were killed. The first several hundred susceptible mice were autopsied to make certain that the growths were a proliferation of the transplant. Autopsy was not considered necessary thereafter, since the results were uniform and the mice were not of sufficient age to develop spontaneous tumors. All mice grossly negative at the conclusion of the experiment were autopsied to detect possible impalpable tumor growths.

## RESULTS

In the $F_1$ generation it is expected on the basis of the genetic theory of tissue transplantation that 100 percent of the animals will show progressive growth of the tumor, since all individuals contain one complete set of genes from each parent. A tissue from one parent stock transplanted into the $F_1$ generation would, therefore, not be recognized as foreign by the host and would not be eliminated.

The two groups of $F_1$ individuals (A × dba; dba × A) would possess exactly the same genetic constitution, all individuals receiving both a dba complex and an A complex. All $F_1$ animals are brown since they have the gene *C* for color and the genetic configuration *CcDdbbaa*.

TABLE 1.—*Inoculation of tumor S91 into $F_1$ generation mice of reciprocal crosses*

| Genetic constitution of mice | Total transplants | Total positives | Unrecorded |
|---|---|---|---|
| | *Number* | *Number* | *Number* |
| A × dba | 79 | [1] 75 | [2] 4 |
| dba × A | 45 | 44 | [3] 1 |

[1] 1 animal negative on first inoculation showed growth of transplant on reinoculation.
[2] 1 lost; 1 negative on first inoculation killed before second inoculation; 1 missing after first inoculation; 1 negative on first inoculation received second but died before results were evident.
[3] Missing after first inoculation.

The results of inoculations into $F_1$ individuals appear in table 1.

Table 1 shows that 100 percent of the mice for which results were recorded grew the transplant. Reinoculations into three animals were necessary because of negative results on the first transplant, of which one showed growth, and no results were available on the other two owing to the death of the animals.

In the $F_2$ generation there is Mendelian segregation of animals according to color, shown in the following scheme:

dba parent *CCddbbaa* A parent *ccDDbbaa*

$F_1$ *CcDdbbaa*

| $F_2$ | | | |
|---|---|---|---|
| 1 | *CCDDbbaa* | 2 *Ccddbbaa* | 2 *ccDdbbaa* |
| 2 | *CCDdbbaa* | 1 *CCddbbaa* | 1 *ccDDbbaa* |
| 2 | *CcDDbbaa* | | 1 *ccddbbaa* |
| 4 | *CcDdbbaa* | | |
| 9 | brown | 3 dilute brown | 4 albino |

The expected color distribution, assuming independent segregation of the genes in the $F_2$ generation, is 9 browns, 3 dilute browns, 4 albinos. Table 2 shows the total $F_2$ observed as compared with the

TABLE 2.—*Color distribution observed and expected in the $F_2$ generation of mice*

| Distribution | Color of $F_2$ mice | | |
|---|---|---|---|
| | Brown | Dilute brown | Albino |
| | *Number* | *Number* | *Number* |
| Observed | 726 | 253 | 341 |
| Expected | 742 | 248 | 330 |

expected numbers on this basis. It will be noted that the observed ratios very closely approximate the expected ratios.

The results of the distribution of susceptible and nonsusceptible animals in the $F_2$ generation will now be considered. The distribution of positive (susceptible) and negative (nonsusceptible) animals in the three color classes after one inoculation is presented in table 3.

Table 3 gives the raw data of the reciprocal crosses as well as the color totals of both types of crosses. The $\chi$ test shows that the percentages of the two reciprocal crosses in the color groups are sufficiently similar to be considered together, although the albinos present a borderline case.

Table 3 shows that the 2 color groups (brown and dilute brown) give similar percentages, but there is a large discrepancy in the ratio of susceptible to nonsusceptible animals within the albino class. When tested statistically, the probability that the observed ratio in the albinos is a random distribution in the sample is less than 1 chance in 17,000 (4.29 times the standard error of difference). This result is highly significant and shows conclusively that added or different elements are involved in the susceptibility of $F_2$ albinos. The results of reinoculation are summarized in table 4.

The total susceptible and nonsusceptible animals in the three color groups calculated on the basis of both inoculations are given in table 5.

Since it was found that ratios of susceptible to nonsusceptible animals in the colored groups differ significantly from that in the albino group, the material is not homogeneous and cannot be treated as one group.

## DISCUSSION

The first fact which is clear from the experimental results is that Mendelian segregation between the two pairs of major color genes involved is regular and not complicated by any differences in relative viability of the classes of $F_2$ young produced. The second fact is that among the colored classes (brown and dilute brown) the number of animals susceptible to inoculation with tumor S91 in the $F_2$ generation bears, to those which are nonsusceptible, a ratio which is suggestive of one Mendelian factor. The third result,

TABLE 3.—*Susceptibility of mice of reciprocal crosses to inoculations with tumor S91*

| Original cross | Color of mouse | Inoculations | Susceptible mice | Nonsusceptible mice | Positive mice | Negative mice |
|---|---|---|---|---|---|---|
| | | *Number* | *Number* | *Number* | *Percent* | *Percent* |
| A × dba | Brown | 382 | 280 | 102 | 73.3 | 26.7 |
| dba × A | do | 319 | 233 | 86 | 73.0 | 27.0 |
| Total or average | | 701 | 513 | 188 | 73.2 | 26.8 |
| A × dba | Dilute brown | 133 | 94 | 39 | 70.7 | 29.3 |
| dba × A | do | 112 | 78 | 34 | 69.6 | 30.4 |
| Total or average | | 245 | 172 | 73 | 70.2 | 29.8 |
| A × dba | Albino | 176 | 113 | 63 | 64.2 | 35.8 |
| dba × A | do | 152 | 83 | 69 | 54.6 | 45.4 |
| Total or average | | 328 | 196 | 132 | 59.8 | 40.2 |

TABLE 4.—*Results of reinoculation of mice of three color classes in the $F_2$ generation with tumor S91*

| Color of $F_2$ mice | Mice negative on first inoculation | Mice reinoculated | | | | |
|---|---|---|---|---|---|---|
| | | Susceptible | Nonsusceptible | Total | Positive | Negative |
| | *No.* | *No.* | *No.* | *No.* | *Pct.* | *Pct.* |
| Brown | 188 | 4 | 84 | 88 | 4.5 | 95.5 |
| Dilute brown | 73 | 4 | 30 | 34 | 11.8 | 88.2 |
| Albino | 132 | 8 | 51 | 59 | 13.6 | 86.4 |

TABLE 5.—*Susceptible and nonsusceptible mice of the three color classes in the $F_2$ generation on the basis of two inoculations with tumor S91*

| Color of $F_2$ mice | Recorded results | Mice from first and second inoculations | | | |
|---|---|---|---|---|---|
| | | Positive mice | | Negative mice | |
| | *Number* | *Number* | *Percent* | *Number* | *Percent* |
| Brown | 701 | 517 | 73.8 | 184 | 26.2 |
| Dilute brown | 245 | 176 | 71.8 | 69 | 28.2 |
| Albino | 328 | 204 | 62.4 | 124 | 37.6 |

and one which is the chief interest in the present discussion, is the fact that among albino segregates in the $F_2$ generation the ratio of susceptible to nonsusceptible animals is significantly different from the ratio obtained in colored $F_2$ mice. Since this difference exists, it requires explanation. Until further experimental data are obtained any explanation must admittedly be of a tentative nature and is advanced as a working hypothesis rather than as an established fact or even a theory in support of which experimental evidence has been obtained.

The first tendency in attempting to explain a result of the type observed is to reason that because the albinos show a different ratio this indicates that there is genetic linkage. Such linkage would have to involve either an extra gene for susceptibility linked with *C*, the factor for color, or a gene for nonsusceptibility introduced and linked to *c*, the gene for albinism. As one attempts to apply such a hypothesis he finds that the difference in susceptibility between the colored and albino $F_2$ animals is not as great as a high degree of linkage would demand, and that, in fact, a majority of albino $F_2$ mice behave as they are expected to under a hypothesis of random Mendelian segregation.

This fact leads one to the assumption that it would be better to have some theoretical explanation that was based on random segregation with some sort of physiologic relationship between the tolerance and growth of a tumor which involves melanin pigment formation and the fact that albinos do not have this ability. Such an explanation can be found in the following theory:

## SUGGESTED EXPLANATION OF GENETIC RESULTS

*C*=color gene
*c*=albino allele
*T*=gene for tumor growth introduced by dba
*t*=its allele
*M*=gene which in cc animals is necessary for support of melanin formation
*m*=its allele, *mmcc* albino animals cannot support growth of S91 melanin tissue

$CcTtMm$=$F_1$ generation formula

Detailed formulas of $F_2$ albinos

| | |
|---|---|
| 1 *TTMMcc*<br>2 *TtMMcc*<br>2 *TTMmcc*<br>4 *TtMmcc* | 9 Susceptible |
| 1 *TTmmcc*<br>2 *Ttmmcc*<br>1 *ttMMcc*<br>2 *ttMmcc*<br>1 *ttmmcc* | 7 Nonsusceptible |

It seems reasonable to suppose that the relationship between a tumor which forms melanin pigment and the somatic cells of an animal unable to do so might be more complicated and open to chemical

discrepancies than would cases in which the body of the host animal had the power to form pigment. If albinos ordinarily lacked, and colored animals ordinarily possessed, a dominant modifying gene which provided, when present in a single "dose," proper chemical surroundings for the development of melanin, we could easily imagine that a relationship existed between this gene *M*, and the main gene for color *C*, which allowed *M* to have an important role in determining the genetic constitution of albinos without expression of this potentiality unless *C* were present.

In one way, the placing of melanin-forming tumor cells in the body of the albino is somewhat similar to introducing into that body cells in which *C* and the hypothesized gene *M* are present and functioning. It is reasonable to assume that, if this were the case, there would be a definite difference between the somatic cells of albinos possessing one or two doses of *M* and those which entirely lacked this gene. If the gene *M* segregated freely and independently of the gene *C*, we should expect approximately three-quarters of the albinos in $F_2$ to have one or two doses of *M*. At the same time, three-quarters of the $F_2$ albinos would have one or two doses of the main gene for the growth of tumor S91 in the same way that the colored $F_2$ animals would have this gene distributed throughout them. Such a situation would produce approximately nine-sixteenths of the $F_2$ albinos with both gene *T* and gene *M* present. Only in these animals would the growth of tumor S91 be possible on the present hypothesis. The result would be a two-factor ratio among albinos, and this is exactly what is approximated in the experimental results.

It would seem reasonable, therefore, to expect that further studies on the physiology of tumor S91 transplanted into albinos, which might be modified by experimental introduction of various chemicals, would provide important evidence as to the possible value of the working hypothesis thus advanced.

## SUMMARY AND CONCLUSIONS

Inoculations with a melanoma, S91, originating in the inbred dba strain of mice show that this tumor grows regularly and successfully in animals of the strain of its origin. It fails to grow in animals of another inbred strain, A albinos.

Inoculation of a large number of hybrids of these strains shows a ratio of susceptible to nonsusceptible animals in $F_2$ colored mice that suggests that one Mendelizing gene is involved in the growth of this tumor.

The albino animals in $F_2$, however, give a different ratio showing that some genetic and/or physiologic differences exist which require explanation.

A tentative working hypothesis is advanced that a gene *M*, necessary for support of melanin formation in albinos, is introduced from the dba strain into the cross and is therefore present in all $F_1$ animals, and that *M* segregates independently in the $F_2$ generation. It is assumed that animals with gene *C*, and the main gene for the growth of the tumor, *T*, will grow the tumor independently of whether or not they have gene *M*.

In the case of albinos (*cc* animals), however, it is assumed that at least one representation of the gene *M* must be present. This hypothesis would satisfactorily fit the experimental figures and would suggest that further physiologic and genetic studies of melanotic tumors transplanted into albinos under various conditions of chemical modification might well prove to be important.

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